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14. ABSTRACT

The Technology/Therapeutic Development Award titled "Intravesical NGF antisense therapy using lipid nanoparticle for interstitial cystitis" evaluates the feasibility of an anti-nerve growth factor (NGF) bladder drug delivery as a treatment for Interstitial Cystitis/Painful Bladder Syndrome (IC/PBS). IC/PBS is a chronic, severely debilitating disease of the urinary bladder which was included as a FY11 Topic Areas of The Peer Reviewed Medical Research Program (PRMRP). The goal of this project is to advance the development of a potential new drug candidate. Specific aims of the project include early stage development, initial manufacture, and animal testing of an experimental liposome NGF-antisense formulations for bladder pain. During the second year of the project, investigators continued development and conducted GMP compliant pilot scale manufacturing product batches, conducted IND relevant study of product formulation and stability, and conducted in-vivo investigation of liposome NGF-antisense formulation in a novel diseased animal model.

15. SUBJECT TERMS
 Interstitial cystitis/painful bladder syndrome, Liposome, Peer Reviewed Medical Research Program, Drug Delivery

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I-1. Introduction

Interstitial cystitis/painful bladder syndrome (IC/PBS) is a poorly understood condition characterized by chronic inflammation of the bladder wall. IC/PBS is associated with bladder symptoms of urinary frequency and urgency, and pain. A recent large-scale epidemiology study on IC is the federally funded RAND Interstitial Cystitis Epidemiology (RICE) study. RICE reports that 3% to 7% of American women have bladder symptoms that could be interstitial cystitis. Their findings correspond to an IC Prevalence of 3,300,000 - 7,900,000 women in the United States, based on a high sensitivity (most inclusive) definition (Berry et al 2011). IC/PBS is a chronic and costly condition of patients and their loved ones.

There are two FDA approved treatments for IC/PBS, although there are significant limitations associated with each. Consequently, there remains a significant need for new therapeutic interventions such as the product candidate of this grant. Industry progress in developing new treatment options for the indication have been slow and limited. The high unmed medical need for this indication warrants multiple treatment approaches by disease stakeholders. The investigators of this project have worked to develop novel treatments for this indication. With this project, these efforts now include local administration of anti-nerve growth factor (NGF) into the bladder, which holds promise for IC/PBS by restricting the effect of administered antisense oligos to the bladder and avoiding systemic safety issues. The goal of this project is to advance preclinical experiments towards the development of a new drug candidate using liposome mediated delivery of NGF for bladder pain. The larger objective of this program is to advance a drug candidate towards FDA regulatory filings for clinical evaluation.

I-2. Keywords

Interstitial cystitis/painful bladder syndrome (IC/PBS), anti-nerve growth factor (NGF), Small interfering RNA (siRNA), liposome, preclinical, in-vivo, urology, bladder, drug delivery, afferent hyperexcitability

I-3. Overall Project Summary

In this project, local therapy targeting NGF production in the bladder for the treatment of bladder pain is contemplated for development and testing. Aims of this project support the investigation of the feasibility and early product development of this new potential treatment option. Aims of the project include formulation development and investigation in preclinical studies. Neither the proprietary name, nor the unique prefix of the non-proprietary name has been assigned to this investigative product, at this time we refer to it as LP-11.

The overall grant summary is: (1) to establish viable NGF antisense formulation for delivery using liposomes (LP-11) locally in the bladder, which diminishes the NGF levels in the urothelium and bladder afferent pathways, for the treatment of bladder pain and C-fiber afferent hyper-excitability to be tested in in rats, and (2) to complete batch manufacturing in parallel with investigation of stability, and preclinical safety to allow an IND submission for Phase I clinical investigation in the United States. To achieve these objectives; the SOW on the following page was developed:

II. Body
II-1. Timeline described in the SOW

	Year 1		Year 2			Year 3		
	0-4mo	5-12mo	13-16mo	17-20mo	21-24mo	25-28mo	29-32mo	33-36mo
AIM 1	☐ Regulatory approval for animal research ☐ Obtain written approval from both IACUC and ACURO	☐ Order animals/ ☐ Prepare protocols/ documentation ☐ Initiate animal study and confirmation of CYP and TNBS	☐ CYP, TNBS treatment ☐ LP-NGF antisense treatment 　o Pain behavior/bladder over-activity assessment ☐Tissue Analysis 　o PCR/ELISA 　o Patch Clamp			Complete animal studies		Animal study data analysis/reporting
AIM 2	☐ Optimize formulation ☐ Manufacture LPs ☐ Analytical method development		☐ Develop GMP LP-NGF antisense manufacture process ☐ cGMP documentation reporting ☐ Manufacture GMP LP ☐ Product specifications/batch release testing ☐ Biosdistribution assessment			☐ Stability Testing ☐ Ex-vivo stress testing		

II-2. Research Accomplishments
Aim 1 (Year 2)
1. *CYP, TNBS treatment, Tissue analysis*
[Accomplishment] Validation of the colitis models and development of the new animal model of chronic cystitis

Building on previous period findings, we continued to examine the mechanisms inducing nociceptive responses in the bladder such as bladder pain behavior and bladder overactivity in rats with experimental colitis.

Overall we have found that; (1) TNBS colitis rats exhibit enhanced freezing behavior that reflects bladder pain, which was suppressed by desensitization of C-fiber afferent pathways by capsaicin pretreatment, (2) 7-14% of L1, L6 and S1 DRG neurons innervate both colon and bladder, (3) the TRPV1 mRNA level is increased in S1 DRG and (4) an increase in MPO activity that reflect the level of neutrophil infiltration was seen in the colon, but not in the bladder. These results indicated that TNBS-induced colitis increased pain sensitivity in the bladder via activation of C-fiber afferent pathways due to colon-to-bladder cross-sensitization (Yoshikawa et al., 2014).

Furthermore, we examined changes in colon and bladder activity after the application of allyl isothiocyanate (AI), a TRPA1 channel activator, into the colon in rats (Furuta et al., 2014). We found that: (1) the intracolonic AI application, which significantly increased mean intracolonic pressure, induces bladder overactivity due to colon-to-bladder cross-sensitization, which is prevented by pretreatment with HC-030031, a TRPA1 inhibitor and (2) Evans blue dye extravasation is significantly increased in the AI-treated inflamed colon and also in the bladder following intracolonic AI treatment. These results indicate that TRPA1 channels expressed in C-fiber afferent pathways greatly contribute to colon-to-bladder cross-sensitization to induce bladder nociceptive responses such as bladder overactivity (Furuta et al., 2014).

Additionally, previous work in phase 1 lent itself to research conducted by the investigators concurrently during this period, which looked at the therapeutic effect of a sensory neuron-specific receptor (SNSR) agonist (BAM8-22) on bladder overactivity in a rat model of cystitis induced by cyclophosphamide (CYP), which was verified as a cystitis model with bladder pain hypersensitivity in the 1st year of this grant, and found that activation of SNSR can suppress CYP-induced bladder overactivity, due to suppression of bladder afferent activity, suggesting that the SNSR is a potential target for the treatment of bladder hypersensitive disorders such as IC/PBS (Honda et al., 2014). Also, using patch-clamp recording techniques, we reported that different types of voltage-gated potassium channel -subunits such as Kv1.4 or Kv4.1/4.3 subunits are involved in the control of neuronal excitability in somatic and visceral afferent neurons including bladder afferent cells in L6-S1 DRG of rats (Yoshimura et al., 2014; Yunoki et al., 2014).

2. *LP-NGF antisense treatment, Tissue analysis*
[Accomplishment] Therapeutic effects of intravesical liposome-NGF antisense treatment

3

During the period investigators examined efficiently of liposome-NGF antisense treatment efficacy for the treatment of bladder nociceptive responses in rat models including TNBS colitis rats (Tyagi et al., 2014, Yoshizawa et al., 2014- AUA abstract). TNBS colitis was produced as described above in rats, and a day before evoking TNBS colitis, either liposomes conjugated with phosphorothioate oligodeoxynucleotide (OND) targeting NGF or saline (vehicle) was instilled to the bladder. At 10 days after TNBS treatment, bladder pain behavior (freezing induced by RTx), bladder activity (cystometry) during 0.1% acetic acid (AA) infusion into the bladder and the mRNA and protein expression of NGF in the bladder mucosa (RT-PCR, ELISA and immunohistochemistry) were evaluated.

In summarizing our findings on investigation of liposome-NGF antisense in a diseased animal during this period we found; (1) in the colitis-saline group, the score of freezing behavior, which represents bladder pain sensation, is higher than that of other groups including the colitis-OND group, (2) the ICI reduction rate after AA instillation into the bladder is significantly higher in the colitis-saline group than that in the colitis-OND group, and (3) The mRNA and protein expression of NGF in the mucosa is significantly higher in the colitis-saline group compared to the colitis-OND group (Kawamorita et al., 2014- AUA & ICS abstracts). These results indicate that the liposome-NGF antisense OND conjugate is effective to suppress the urothelial NGF expression and inhibit bladder nociceptive responses such as bladder pain behavior and bladder overactivity in TNBS colitis rats.

Aim 2 (Year 2)
1. *Develop GMP LP-NGF antisense manufacture process, cGMP documentation/reporting, and manufacture GMP LP*
[Accomplishment]
Preliminary pilot production of LP-11 was explored in the 1st period of the grant and showed its viability using only slightly modified facilities. The goal of this period's effort was to increase the scale and quality of pilot production of LP-11. Product candidate material that resulted from manufacturing batches during this year were using in experiments described in this report and created a small surpluss of the material for future investigation.

The manufacture of LP-11 parameters for lyophilization production of liposomes have been established to ensure quality final product. Manufacturing of GMP liposomes has been expanded during the second period of this project to include the incorporation of the NGF antisense. Standard operating procedures (SOPs) have been written in accordance Lipella's existing quality systems program to further GMP compliance of the liposomal associated NGF.

During the period we made several improvements to the manufacturing process to both enhance production efficiency while also increasing quality assurance and control for final products. These improvements allow production to occur in a more streamlined and efficient process. Test materials were manufactured to confirm and ensure final product quality is maintained with the procedure improvements including removal of unnecessary steps in the manufacturing process as well as finding suitable replacements for specific steps in the process for tasks that were previously more apt to quality issues and gaps in processing time. This included a modification to the process, which involved increasing the secondary freezing stage in the lyophilization stage that takes place in the tray freezer to 10 hours at -40C to as a replacement for the primary freezing step in the shell freezer at -40C. This change allowed for more consistent treatment of production materials as it reduced lag time for between filling and freezing individual batches of vials.

Overall, at the completion of this project our objective is to achieve a manufacturing protocol and capability that is compliant with the hopeful introduction of LP-11 into FDA phase I clinical investigation (and beyond to phase II&III) and that our process results in a formulation that is reasonably safe to proceed with these plans from a production perspective. During this period we are more complaint with clinical studies level quality at

this time, and during the time of this grant, investigators will continue to refine the manufacturing process for this purpose. In the next period this will include enhancement and validation of parts of the SOP.

3. *Product specifications/ Biodistribution assessment*

Development has been performed during the grant period to work on a method and for determining the extent of encapsulation or entrapment volume of the siRNA antisense and the liposomes. Investigators in collaborating with a CRO (Ricerca Biosciences, LLC) studied the total oligonucleotide content in phospholipid-based Lipo-siRNA formulation using High Performance Liquid—Strong Ion Exchange chromatography (HPLC-SAX) with UV detection.

Analysis of Formulation on As-Is Basis was performed first to determine signal of total oligonucleotide in the formulation. LP-11 powder was reconstituted in water for injection and were used to attempt the determination of the Oligonucleotide. Samples were analyzed on as-is basis and after addition of authentic Oligonucleotide. Triton X-100 was used to destroy (disrupt, dissolve) the micellar structure yielding a clear homogeneous solution

Additionally, samples of Lipo-siRNA reconstituted formulation were analyzed on as-is basis and after addition of Oligonucleotide authentic substance for verification of recovery (**Figure 1).**
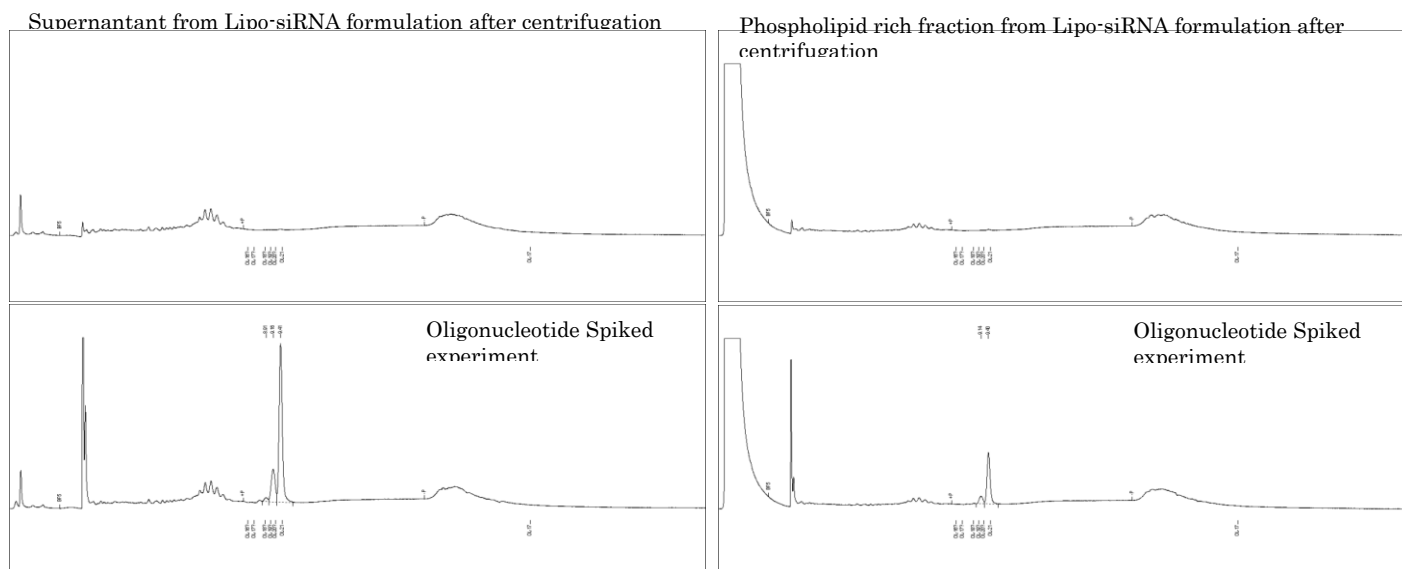
Figure 1: UV chromatograms of supernatant and phospholipid-rich fraction after centrifugation of the Lipo-siRNA

Work on this element of the product application has been useful in helping to establish a better manufacturing process for the product. These period 2 findings suggested that the Oligonucleotide substance is in degraded form (e.g. smaller oligomers) in the LP-11 samples studied. This could be degrading the performance of the product and requires additional study and refinement which is ongoing. Further investigation will be done to determine particular manufacturing steps that may be critical for the siRNA antisense material or whether the addition of a protectant into the formulation would be advantageous and feasible. Work proposed in the SOW period 3 including stress testing of the product conform to this objective.

III. Research accomplishments
- Validation of the colitis models and development of the new animal model of chronic cystitis
- Successful LP-NGF antisense treatment evaluation in animal model
- Development and pilot scale LP-11 manufacture
- Analysis on encapsulation efficiency of liposomes

IV. References

Berry SH, Elliot MN, Suttorp M, Bogart LM, Stoto, Eggers P, Nyberg L, Clemen, JQ (2011) Prevalence of Symptoms of Bladder Pain Syndrome/Interstitial Cystitis Among Adult Females in the United States. J Urol. 2011 August ; 186(2): 540–544. doi:10.1016/j.juro.2011.03.132.

V. Conclusions

In the second year of the project, we continuing the progression of LP-11 manufacturing, studied the product formulation, conducted IND relevant studies, and refined an animal model to generate preclinical animal data. These results support the continued justification of the development program into the next project period. Study on encapsulation revealed additional formulation work to be resolved to create a more effective product. Additional manufacturing refinement steps were are also identified as improvements to make to improve the potential regulatory assessment of the process. In the next period our goal is to complete liposome-NGF antisense efficacy investigation of the disease model, and complete the reminder of the originally proposed SOW. Development of LP-11 is exciting. We have begun assembling materials for anticipated-initial outreach to the FDA. We hope work on year 3 can build upon previous findings and add value to the LP-11 program.

VI. Publications and Reportable Outcomes

Refereed articles (Year 2 only):
1. Yoshimura, N., Oguchi, T., Yokoyama, H., Funahashi, Y., Yoshikawa, S., Sugino, Y.: Kawamorita, N., Kashyap, M.P., Chancellor, M.B., Tyagi, P., Ogawa T.: Bladder afferent hyperexcitability in bladder pain syndrome/interstitial cystitis (BPS/IC). International Journal of Urology (supplement), 21 (Suppl 1), 18–25, 2014.
2. Tyagi, P., Kashyap, M.P., Kawamorita, N., Yoshizawa, T., Chancellor, M., Yoshimura, N.: Intravesical liposome and antisense treatment for detrusor overactivity and interstitial cystitis/painful bladder syndrome. ISRN Pharmacology, 2014, Article ID 601653, 12 pages (open access at http://dx.doi.org/10.1155/2014/601653).
3. Furuta, A., Suzuki, Y., Naruoka, T., Asano, K., Egawa, S., Yoshimura, N.: Cross-sensitization mechanisms between colon and bladder via transient receptor potential A1 stimulation in rats. International Urogynecology Journal, 25: 1575–1581, 2014.
4. Yoshikawa, S., Kawamorita, N., Oguchi, T., Funahashi, Y., Tyagi, P., Chancellor, M.B., Yoshimura, N.: Pelvic organ cross-sensitization to enhance bladder and urethral pain behaviors in rats with experimental colitis. Neuroscience, in press, 2014.
5. Yunoki, T., Takimoto, K., Kita, K., Funahashi, Y., Takahashi, R., Matsuyoshi, H., Naito, S., Yoshimura, N.: Differential contribution of Kv4-containing channels to A-type, voltage gated potassium currents in somatic and visceral dorsal root ganglion neurons. Journal of Neurophysiology, in press, 2014.

Published abstracts:
1. Tyagi. P., Kashyap, M., Chancellor, M.B., Yoshimura, N: Investigation into constitutive expression of nerve growth factor in bladder by bladder wall injection of NGFantisense. 112th Annual Meeting AUA, MP76-01, Orlando, FL, May 16-22, 2014.
2. Kawamorita, N., Yoshikawa, S., Arai, Y., Tyagi, P., Chancellor, M.B., Yoshimura, N.: Therapeutic effect of antisense oligonucleotide (OND) targeting nerve growth factor (NGF) on bladder hypesensitivity in rat with experimental colitis. 112th Annual Meeting AUA, PD9-01, Orlando, FL, May 16-22, 2014.
3. Yoshizawa, T., Tyagi, P., Chancellor, M.B., Takahashi, S., Yoshimura, N.: Therapeutic effects of intravesical application of liposome conjugated with antisense oligonucleotide targeting nerve growth factor on neurogenic detrusor overactivity in rats with spinal cord injury. 112th Annual Meeting AUA, MP1-17, Orlando, FL, May 16-22, 2014.
4. Kawamorita, N., Yoshikawa, S., Tyagi, P., Chancellor, M.B., Yoshimura, N.:Liposome-based intravesical therapy targeting nerve growth factor (NGF) ameliorates bladder hypersensitivity in rats with experimental colitis. 44th Annual meeting of the International Continence Society, Abstract. No. 372, Rio de Janeiro, Brazil, October 20-24, 2014. Neurourology and Urodynamics (2014) 33: 829-831.

Review Article

Bladder afferent hyperexcitability in bladder pain syndrome/interstitial cystitis

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Abbreviations & Acronyms
BPS = bladder pain syndrome
CREB = cyclic adenosine monophosphate-responsive element binding protein
CYP = cyclophosphamide
DRG = dorsal root ganglion
FDA = Food and Drug Administration
GlyT = glycine transporters
HSV = herpes simplex virus
IC = interstitial cystitis
IFN-γ = interferon-γ
IL = interleukin
NGF = nerve growth factor
TNF-α = tumor necrosis factor-α
TNF-αsR = tumor necrosis factor-α soluble receptors
Trk = tyrosine kinase receptor
TRP = transient receptor potential

Abstract: Bladder pain syndrome/interstitial cystitis is a disease with lower urinary tract symptoms, such as bladder pain and urinary frequency, which results in seriously impaired quality of life of patients. The extreme pain and urinary frequency are often difficult to treat. Although the etiology of bladder pain syndrome/interstitial cystitis is still not known, there is increasing evidence showing that afferent hyperexcitability as a result of neurogenic bladder inflammation and urothelial dysfunction is important to the pathophysiological basis of symptom development. Further investigation of the pathophysiology will lead to the effective treatment of patients with bladder pain syndrome/interstitial cystitis.

Key words: afferent pathway, bladder pain syndrome, cytokine, interstitial cystitis, nerve growth factor.

Introduction

BPS/IC is a debilitating chronic disease characterized by suprapubic pain related to bladder filling, coupled with additional symptoms, such as increased day- and night-time urinary frequency, without proven urinary infection or other obvious pathology.[1–3] Although the symptoms presented might appear similar to those of a urinary tract infection, urine culture shows no underlying infection and there is no response to antibiotic treatment.[4,5] It has been estimated that the prevalence of BPS/IC range from 1.2 per 100 000 population and 4.5 per 10 000 females in Japan.[6] Although the etiology is unknown, theories explaining the pathology of BPS/IC include altered barrier lining, afferent and/or central nervous system abnormalities, possible contribution of inflammatory or bacterial agents and abnormal urothelial signaling. Figure 1 presents the proposed pathogenesis of BPS/IC in which there is bladder insult and damage to the urothelial layer that, for example, allows substances in urine, such as potassium, to leak into the suburothelium and to prompt a cascade of events as well as cross-talk with other pelvic organs, each contributing to bladder inflammation and pain.[1] In the present review, among these various etiological factors, we focus on the potential mechanisms underlying increased excitability of bladder afferent pathways in BPS/IC.

Plasticity of sensory pathways inducing afferent hyperexcitability

Clinical findings

As a local mechanism inducing afferent sensitization and hyperexcitablity, it has been proposed that urothelial dysfunction that can increase the amount of urothelially-released substances, such as adenosine triphosphate and nitric oxide, and subsequent neurogenic inflammation associated with mast cell activation can lead to changes in the properties of bladder afferent pathways, resulting in increased pain sensation associated with BPS/IC (Fig. 1).[1] Pain is a defining characteristic of BPS/IC. One mechanism by which pain is induced is postulated to involve chronic tissue inflammation that can lead to functional changes in C-fiber afferents.[7] Hyperactivity and emergence of mechanosensitivity of C-fiber afferents might lead to pain sensation in response to normal non-noxious distension of the bladder. Indirect evidence for this postulate comes from histological analysis of bladders from patients with BPS/IC, which showed marked edema, vasodilation, proliferation of nerve fibers and infiltration of mast cells.[8] Also, increased afferent
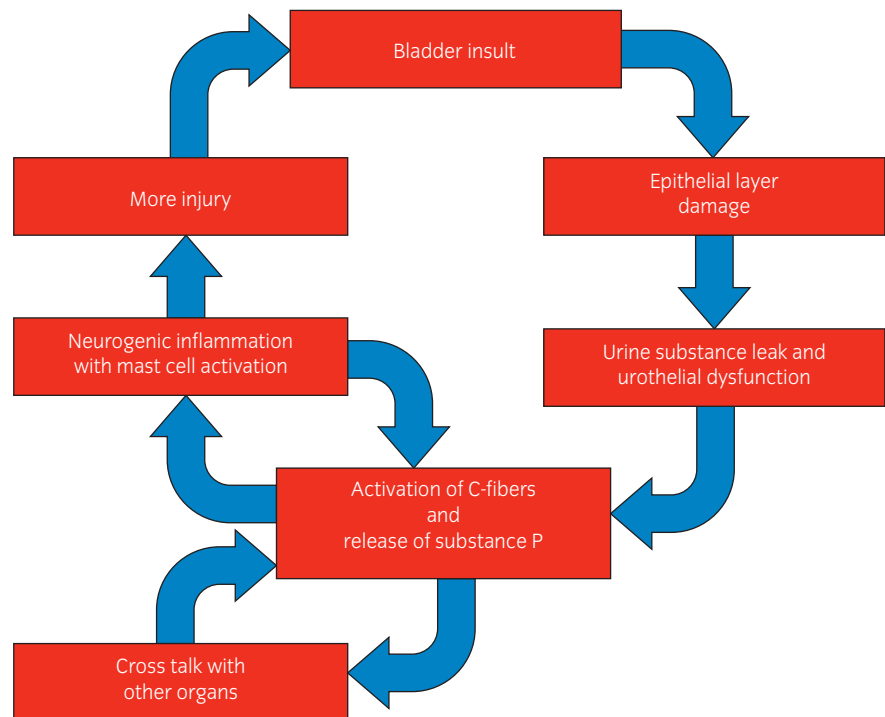
Fig. 1 Proposed pathogenesis of BPS/IC.

activity is evidenced by a statistically significant increase in the number of nerve fibers expressing substance P, which is a major neurotransmitter of C-fiber afferents, and the mRNA levels of substance P receptors (neurokinin-1) in the mucosa of the bladder in patients with IC.[9] It has also been reported that C-fiber desensitization by means of intravesical application of high-dose capsaicin and resiniferatoxin is effective for treating painful symptoms in BPS/IC patients,[10,11] although a prospective, randomized clinical trial using intravesical resiniferatoxin application was not effective in patients with IC.[12] More recently, intravesical application of alkalized lidocaine, a local anesthetic, has been shown to provide sustained amelioration of symptoms of BPS/IC in a placebo-controlled, randomized clinical study.[13]

Overall, sensitization and enhanced excitability of afferent pathways innervating the lower urinary tract is likely to be involved in the pathophysiological basis of BPS/IC (Fig. 2).

Basic research findings

The mechanisms inducing afferent sensitization associated with cystitis are further investigated using various animal models of bladder inflammation, in which increased urinary frequency is initiated by sensitizing mechanosensitive afferents and/or recruitment of afferents normally unresponsive to mechanical stimulation.[14–17] Additionally, pro-inflammatory agents, such as prostaglandin E2, serotonin, histamine and adenosine; as well as neurotrophic factors, such as NGF, can induce functional changes in C-fiber afferents that can lead to these relatively unexcitable afferents becoming hyperexcitable.[18–21] Tissue inflammation in the bladder can also induce changes in the expression of various neurochemical markers in the bladder and bladder afferent pathways. Using an animal model of chronic bladder inflammation induced by cyclophosphamide, it

has been reported that the expression of nitric oxide synthase,[22] growth-associated protein,[23] pituitary adenylate cyclase-activating polypeptide,[24] neuropeptides such as substance P,[25] protease activated receptors,[26] cyclooxygenase-2 and prostaglandins are increased in afferent neurons in lumbosacral DRG, innervating the bladder in rats with CYP-induced chronic cystitis. Thus, it is likely that chronic bladder inflammation can induce various changes in the expression of inflammation-related proteins/receptors in the bladder and bladder afferent pathways, and that these changes might contribute to afferent neuroplasticity, leading to pain symptoms in BPS/IC (Fig. 2).

Organ cross-talk and BPS/IC

It has been demonstrated that BPS/IC patients often exhibit high comorbidity of other visceral pain syndromes, such as irritable bowel syndrome.[27] Recent animal studies have shown that interactions with bidirectional cross-sensitization can occur between the bladder and intestine or female reproductive organs. For example, chemically-induced colitis in rats induces urinary frequency, bladder afferent fiber hyperexcitability and mast cell infiltration in the bladder,[28–30] and release of neuropeptides such as substance P and calcitonin gene-related peptide in the bladder,[31] and increases excitability of bladder afferent neurons.[32] In addition, 17% of rat DRG neurons are double-labeled after dye injection into the bladder and colon wall of the rat, showing the existence of dichotomized afferents innervating multiple visceral organs that could contribute to cross-organ sensitization of visceral organs.[33] In addition, another potential site for organ cross-talk could be found at the spinal cord level, because acute colitis in rats reportedly sensitizes spinal neurons receiving input from the bladder in the dorsal horn of the spinal cord.
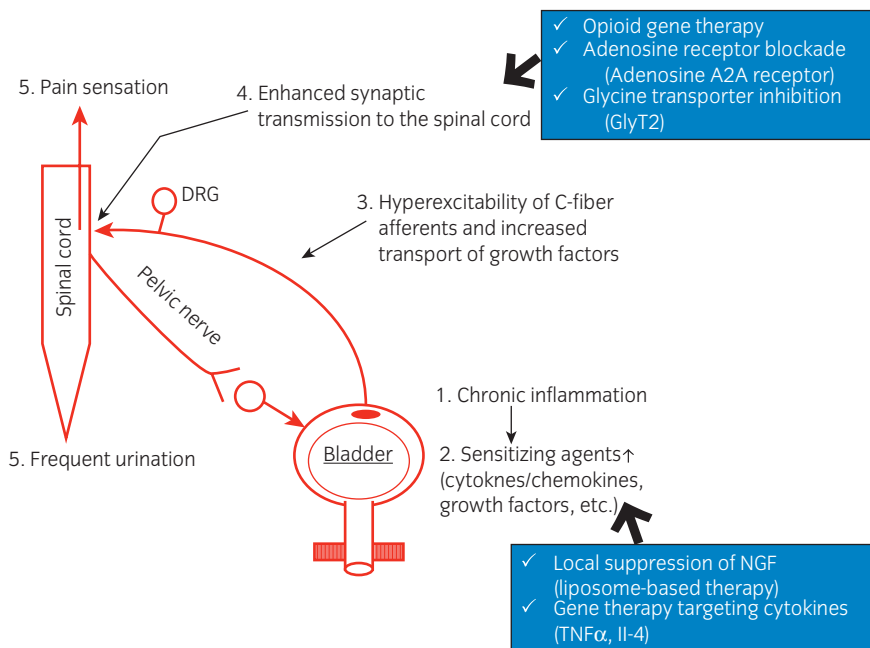
Fig. 2 Summary of the events involved in chronic inflammation of the bladder and hyperexcitability of C-fiber bladder afferent pathways. The events that occur after chronic bladder inflammation (1) are shows in sequential numbers (2–5). Potential therapeutic modalities targeting enhanced synaptic transmission at the spinal cord level or inflammatory changes in the bladder are also shown in the boxes.

Neurotrophic factor and afferent hyperexcitability

Neurotrophic factors, such as NGF, can be synthesized by various inflammatory cells, such as lymphocytes and mast cells, and might function as a prominent factor linking hypersensitivity and development of pain with various inflammatory states, including airway, allergic or neurogenic inflammation including BPS/IC.[34,35] In patients with IC, neurotrophins, including NGF, neurotrophin-3 and glial-derived neurotrophic factor, have been detected in the urine.[36] Increased expression of NGF is also present in bladder biopsies from women with IC.[37] Thus, target organ–neural interactions mediated by an increase of neurotrophins in the bladder and increased transport of neurotrophins to the neuronal cell bodies in afferent pathways might contribute to the emergence of bladder pain in BPS/IC.[38] Furthermore, the monoclonal NGF neutralizing antibody, tanezumab, has been included in the clinical testing program, and encouraging results of the phase two efficacy study have been presented. Tanezumab improved the self-reported pain score and urgency episode frequency 6 weeks after a single intravenous injection 200 μg/kg in a cohort of 68 patients with BPS/IC.[39] Tanezumab had no significant effect on micturition frequency or mean voided volume per micturition. Among adverse effects, abnormal peripheral sensation symptoms, such as paresthesia and hyperesthesia, were more common in the treatment arm when compared with a placebo.[39] Although clinical studies were put on hold after the reports of bone necrosis requiring total joint replacements in clinical trials for ostheoarthritis (http://www.clinicaltrials.gov), proof-of-concept evidence has been provided for the effectiveness of systemic intervention in the NGF system in the treatment of BPS/IC.

In basic research using a rat model of cyclophosphamide-induced chronic cystitis, increased expression of neurotrophic growth factors, such as NGF, brain-derived neutotrophic factor and ciliary neurotrophic factor, in the bladder as well as phosphorylation of tyrosine kinase receptors (TrkA, TrkB) in bladder-innervating afferent neurons has been documented as direct evidence for neurotrophin-mediated signal transduction in chronic bladder inflammation. In addition, the enhanced neurotrophic factor mechanisms were also associated with increased phosphorylated CREB in bladder afferent neurons, and a subpopulation of phosphorylated-CREB-positive cells co-expressed phophorylated-Trk in rats with chronic cystitis.[40] Furthermore, resiniferatoxin, a C-fiber neurotoxin, reduced CYP-induced upregulation of phosphorylated-CREB in DRG, suggesting that cystitis can be linked with an altered CREB phosphorylation in capsaicin-sensitive C-fiber bladder afferents.[40] These results suggest that upregulation of phosphorylated-CREB might be mediated by a neurotrophin/Trk signaling pathway, and that CREB phosphorylation could play a role as a transcription factor in lower urinary tract plasticity induced by cystitis. Previous studies also showed that exogenous NGF can induce bladder nociceptive responses and bladder overactivity in rats when applied acutely into the bladder lumen[15,41] or chronically to the bladder wall or intrathecal space.[42] Turpentine oil-induced cystitis instillation exaggerated micturition reflexes and visceral hyperalgesia within hours, resembling symptoms of human BPS/IC, and was associated with increased NGF mRNA level in the bladder.[43] Importantly, under these experimental conditions, hyperexcitability of the bladder[15] and viscerosomatic hyperalgesia[44] can be prevented with recombinant fusion protein of extracellular TrkA domain with the fragment crystallizable portion of immunoglobulin G to neutralize NGF, confirming the causal role of NGF. Along with modulation of NGF bladder levels, upregulation and a shift in spatial expression of both NGF receptors in nerves was reported in CYP cystitis. After the CYP injection, TrkA seems to be upregulated in DRG,[45] the major pelvic ganglion and bladder smooth muscle, while being

downregulated in the urothelium.[46] Similarly, p75[NTR] immunoreactivity was markedly increased in bladder nerve fibers, Schwann cells, the spinal cord and bladder afferent neurons.[47,48] Focusing on BPC/IC-related hyperalgesia, rather than bladder hyperactivity, Guerrios *et al*. found that CYP-induced peripheral hyperalgesia to mechanical stimulation in mice was abolished by NGF antiserum or k252a, a non-specific TrkA antagonist, but did not affect edema in the bladder wall, leukocyte infiltration or hemorrhage.[49,50] The role of the low-affinity p75[NTR] receptor was also implicated in a study by showing that blockade of p75[NTR] with monoclonal antibody, effectively reducing p75 immunoreactivity in the bladder, induces, rather than limits, hyperreflexia both in control and CYP cystitis rats, as reflected by increased frequency of voiding and reduced intercontraction intervals.[47] Additionally, prevention of NGF binding to p75[NTR] with PD90780 increased voiding frequency with decreased voiding volume, intercontraction intervals, increased threshold, baseline and micturition pressure in cystitis rats.

Taken together, there exists some evidence favoring intervention in the NGF system as a viable therapeutic target ameliorating specifically sensory and painful symptoms associated with BPS/IC. As aforementioned, a recent clinical trial showed that systemic administration of monoclonal human NGF antibodies reduces bladder pain symptoms in BPS/IC patients; however, generalized blockade of NGF activity at sites other than the bladder using systemic anti-NGF antibodies was associated with the incidence of paresthesia, hypoesthesia and bone necrosis.[39] Thus, the site-specific reduction of NGF would be desirable to reduce the intrinsic toxicity from systemic blockade of NGF. In this regard, we recently reported that treatment with intravesical liposomal antisense suppresses NGF expression in the urothelium, as well as bladder overactivity and chemokine upregulation using rats with acetic acid induced bladder overactivity.[51] Thus, local suppression of NGF in the bladder using intravesical liposome-based delivery techniques could be an attractive approach for BPS/IC treatment, which can avoid systemic side-effects that might be associated with non-specific blockade of NGF expression (Fig. 2).

Potential modalities targeting hyperexcitable bladder afferent pathways for the treatment of BPS/IC

Sensitization of bladder afferent pathways, especially of the C-fiber population, and a subsequent increase in sensory processing in the spinal cord have been proposed as important mechanisms inducing pain/irritative symptoms associated with BPS/IC.[52] Thus, therapies aiming to reduce sensory processing in the spinal cord, which is increased as a result of afferent hyperexcitability, could be effective for reducing symptoms in BPS/IC patients (Fig. 2). In the following sections, we discuss the potential targets that can suppress bladder afferent activity for the treatment of BPS/IC, based on recent research results including ours.

Opioids

Opioids are the abundantly-expressed inhibitory neurotransmitter. Patients with BPS/IC often have severe and refractory

bladder pain that is resistant to non-steroidal anti-inflammatory drugs, requiring long-acting opioids.[53] However, the use of systemic opioid therapy, such as morphine or oxycodone, has been limited because of its untoward side-effects, tolerance and dependency. Enkephalins, which are a subfamily of endogenous opioids, are expressed in bladder afferent and efferent pathways to inhibit micturition.[54] Enkephalinergic mechanisms in the brain and spinal cord also have inhibitory effects on the micturition reflex, and exogenous enkephalins or opiate drugs applied to the sacral spinal cord can suppress micturition.[55]

Replication-deficient HSV vectors encoding preproenkephalin, one of three genes that encode endogenous opioid peptides, injected into the bladder wall showed reductions in bladder overactivity and nociceptive behavior induced by intravesical application of capsaicin, whereas vector-mediated expression of enkephalin did not affect normal voiding.[56,57] These results provide the proof of concept for a new gene therapy approach to enhance endogenous opioid mechanisms and reduce systemic side-effects for the treatment of bladder hypersensitive disorders, such as BPS/IC.

Another study reported the antinociceptive effect of human pro-opiomelanocortin (POMC),[58] which encodes β-endorphin, another endogenous opioid peptide, which activates μ-opioid receptors on sensory neurons and inhibits pain in the inflamed tissue.[59,60] Intravesical instillation of acetic acid during cystometrograms shortened the intercontraction interval in control animals, but not in rats treated with POMC genes delivered by gene gun into the bladder wall. The effects of the POMC gene gun injection were reversed by an intramuscular injection of the opiate receptor antagonist, naloxone, supporting an opioid receptor-mediated analgesic mechanism of action.[58]

Overall, opioid gene therapy targeting the bladder and bladder afferent pathways could be an effective modality for the treatment of intractable pain symptoms of BPS/IC while reducing systemic side-effects.

Adenosine receptors

Adenosine is a neurotransmitter that exerts numerous physiological effects in many organs. Recently, a number of studies showed that activation of A1 receptors produces antinociception in several pain models,[61] and inhibition of A2A receptors attenuates nociceptive responses in mouse models of somatic pain.[62,63] Our recent study has also shown that, in rats with irritated bladders induced by intravesical acetic acid application, intravenous or intrathecal ZM24138 (A2A receptor-antagonist) significantly reduces bladder overactivity with significantly greater effects compared with those in untreated rats.[52] These results show that adenosine A2A receptor-mediated excitatory mechanisms are enhanced in the spinal cord after C-fiber bladder afferent stimulation. Thus, adenosine A2A receptor antagonists might be effective for the treatment of bladder hypersensitive disorders, such as BPS/IC, in which C-fiber afferent function is enhanced (Fig. 2).

GlyT system

Glycine, one of the major inhibitory neurotransmitters in the central nervous system, is well known to have a role in the control of spinal nociceptive pathways[64] and lower urinary tract

function in both physiological and pathological conditions.[65–67] The extracellular concentration of glycine at synapses is regulated by two types of Na^+/Cl^- dependent GlyT: GlyT1 and GlyT2.[68] GlyT1 is widely distributed in the central nervous system and predominantly expressed in glial cells near both excitatory and inhibitory neurons, whereas GlyT2 is specifically distributed in the spinal cord, cerebellum and brainstem, and localized in the presynaptic terminals of inhibitory glycinergic neurons.[68–70]

Recent studies have suggested a therapeutic potential for GlyT inhibitors in the treatment of acute/chronic pain.[71,72] Therefore, we have examined the effects of GlyT inhibitors on bladder overactivity and pain behavior in rats.[73] Cystometrogram parameters were used to determine the effects of selective GlyT2 inhibitors administered intrathecally to CYP-treated rats under urethane anesthesia. In addition, intravesical administration of resiniferatoxin was used to induce nociceptive responses in a behavioral study in conscious rats. A selective GlyT2 inhibitor (ALX-1393), but not a GlyT1 inhibitor (sarcosine), produced significant increases in intercontraction interval and micturition pressure threshold in CYP-treated rats. These effects were completely reversed by the glycine receptor antagonist, strychnine. The GlyT2 inhibitor also significantly suppressed nociceptive behaviors in a dose-dependent manner. Compared with GlyT1, mRNA levels of GlyT2 were 23-fold higher in the dorsal spinal cord. Results show that GlyT2 is primarily involved in the clearance of extracellular glycine in the spinal cord, and that GlyT2 inhibition leads to reduction of CYP-induced bladder overactivity and pain behavior.

Therefore, inhibition of GlyT2, but not GlyT1, could be a novel therapeutic modality for the treatment of bladder hypersensitive disorders, such as BPS/IC, without affecting the glycinergic mechanism in the brain (Fig. 2).

Inflammatory and immunogenic mechanisms of BPS/IC

Cytokine and chemokine expression and BPS/IC

Although the etiology of BPS/IC is not fully understood, bladder inflammation associated with the production of inflammatory cytokines/chemokines has been proposed as a potential cause of pathogenesis of the disease to induce afferent hyperexcitability.[74,75] In previous studies, cytokines and chemokines, such as IL-2, IL-6, IL-8 and TNF-α, were significantly increased in BPS/IC patients' bladder tissue and urine than in controls, suggesting that these cytokines might represent specific markers of BPS/IC.[74,76,77] In contrast, IL-4 is a prototypical anti-inflammatory cytokine, which is known to inhibit secretion of inflammatory cytokines, such as IL-1β, TNF-α and IL-6.[78–80] BPS/IC patients have shown lower levels of IL-4 that increased after treatment with a drug (suplatast tosilate) that altered the BPS/IC phenotype and symptoms in these BPS/IC patients,[80] suggesting that IL-4 could provide a desired anti-inflammatory effect that can alter the overall cytokine profile and subsequent recruitment of T cells and mast cells.

Chemokines also play a pivotal role in the immune response, leading the recruitment of leukocytes to inflammation, tumor growth, angiogenesis and organ sclerosis.[81] CXCR3 and its associated ligands, monokine induced by IFN-γ (MIG/CXCL9), IFN-γ-inducible protein (IP-10/CXCL10), and IFN-γ-inducible T cell α-chemoattractant (I-TAC/CXCL11) are involved in the regulation of autoimmune disorders of endocrine glands. CXCR3 was initially identified on activated T cells and its expression was associated with T helper-mediated immune response.[82,83] CXCR3 expresses in epithelial cells, endothelial cells and vascular pericytes.

It has been reported that after IFN-γ stimulation, endocrine epithelial cells secrete CXCL10, which in turn recruits type 1 T helper lymphocytes expressing CXCR3 and secreting IFN-γ, thus perpetuating autoimmune inflammation, suggesting that the chemokines play an important role in endocrine autoimmunity.[81] Sakthivel *et al*. showed that serum levels of CXCL9, CXCL10 and CXCL11 were increased in patients with IC.[84] They also showed that the number of CD4+ T cells, mast cells, natural killer cells, and natural killer T cells were increased at systemic (spleen) and peripheral (urinary bladder and iliac lymph nodes) sites in a mouse model of CYP-induced cystitis. Importantly, CXCL10 blockade attenuated these increases caused by CYP.[84] We also investigated genes responsible for ulcerative IC among over 40 000 genes using microarray analysis.[76] We have identified 564 probes that were significantly expressed ($P < 0.001$) in mRNA by more than fourfold compared with the control group by using the volcano plot analysis. Further ingenuity pathways analysis of these genes showed the top three functions, such as cell-to-cell signaling and interaction, hematological system development and function, and inflammatory disease. In particular, we confirmed the increases in mRNA expression of several genes in the bladder from ulcerative IC, including CXC3-binding chemokines (CXCL9, 10 and 11) and TNFSF14. CXCR3 and its binding chemokines are upregulated in the bladder urothelium of ulcerative BPS/IC patients, which might inhibit the bladder urothelial growth and induce the ulcerative changes in the bladder. In addition, it should be noted that the European Society for the Study of Interstitial Cystitis proposed that Hunner's " 'ulcer' " is not a typical chronic ulcer, but rather a distinctive inflammatory lesion presenting a characteristic deep rupture through the mucosa. The term, "Hunner's ulcer", should be replaced by "Hunner's lesion", and the patients with Hunner's lesion should be classified into the European Society for the Study of Interstitial Cystitis type 3.[85] Our recent study using multiplex analysis of 23 cytokines/chemokines with a multiple antigen bead assay also investigated the cytokine/chemokine profile in bladder tissue and urine of BPS/IC patients, and found that vascular cell adhesion molecule-1 and intercellular adhesion molecule-1 were useful for the discrimination of both tissue and urine samples of BPS/IC patients from control patients.[86]

Gene therapy for modulating the cytokine level

TNF-α is a pro-inflammatory mediator that initiates inflammatory reactions of the innate immune system: induction of other cytokine production, activation and expression of adhesion molecules, and stimulation and recruitment of inflammatory cells.[87] Additionally, it is essential in the development of

nociception, not just in inflammatory pain, but also in neuropathic pain.[88] Many studies have reported that TNF-α influences pain sensation in the peripheral tissue and the spinal cord.[89] When TNF-α activity is neutralized using anti-TNF-α antibody or TNF-αsR, the development of nociception is suppressed, as has been observed in several rat pain models.[89–91]

Recently, gene therapy of TNF-αsR using replication-deficient HSV vectors has been investigated for bladder pain and urinary frequency using a rat model of chemically-induced cystitis.[92] Although TNF-α mRNA in the bladder was upregulated by the intravesical administration of resiniferatoxin (a TRPV1 receptor agonist), the increase in TNF-α protein levels was suppressed in TNF-αsR-expressing vector-treated rats, showing that HSV vector-delivered TNF-αsR neutralized TNF-α proteins increased by bladder irritation. The suppression of TNF-α protein leads to the downregulation of IL-1β and IL-6, as well as a reduction in myeloperoxidase activity in the resiniferatoxin-treated bladder. Moreover, TNF-α blockade reduced pain sensation and bladder overactivity induced by intravesical instillation of resiniferatoxin.

Another approach investigated for bladder pain is the HSV vector-mediated delivery of IL-4 to the bladder and bladder afferent pathways. IL-4 is a prototypical anti-inflammatory cytokine, known to inhibit the secretion of inflammatory cytokines, such as IL-1β, TNFα and IL-6.[78,79] In contrast to TNFα or IL-6, IL-4 is decreased in the urine of BPS/IC patients, and then increases after the treatment of suplatast tosilate, an anti-allergic drug.[80] In rats, IL-4 expression after HSV vector administration to the plantar foot surface reduced in nociceptive behaviors after painful insult.[93] The effect of IL-4 delivered by HSV vector on bladder overactivity and nociceptive behaviors has been evaluated previously.[94] The IL-4 protein level was increased in the bladder and L6 DRG in IL-4-expressing HSV-injected rats, and myeloperoxidase activity and IL-1β were decreased in the bladder of the rat with chemically-induced cystitis, in which bladder overactivity and nociceptive behaviors were also suppressed.[94]

Overall, gene therapy targeting cytokine production in the bladder could be a new modality for the treatment of bladder hypersensitive disorders, such as BPS/IC, which is associated with increased production of inflammatory cytokines/chemokines (Fig. 2).

Conclusion

Although various etiologies of BPS/IC have been proposed as described in the present review, no one pathological process has been identified in every patient with BPS/IC. Thus, it is likely that the syndrome of BPS/IC could have multiple etiologies, all of which result in similar clinical manifestations (Fig. 1), and that afferent hyperexcitiablity would be an important pathophysiological basis of BPS/IC, for which various therapeutic modalities could be developed (Fig. 2).

Acknowledgment

Conflict of interest

None declared.

References

1 Chancellor MB, Yoshimura N. Treatment of interstitial cystitis. *Urology* 2004; **63** (3 Suppl 1): 85–92.

2 Homma Y, Ueda T, Ito T, Takei M, Tomoe H. Japanese guideline for diagnosis and treatment of interstitial cystitis. *Int. J. Urol.* 2009; **16**: 4–16.

3 Hanno PM, Burks DA, Clemens JQ *et al.* AUA guideline for the diagnosis and treatment of interstitial cystitis/bladder pain syndrome. *J. Urol.* 2011; **185**: 2162–70.

4 Parsons CL, Benson G, Childs SJ, Hanno P, Sant GR, Webster G. A quantitatively controlled method to study prospectively interstitial cystitis and demonstrate the efficacy of pentosanpolysulfate. *J. Urol.* 1993; **150**: 845–8.

5 Hanno PM, Landis JR, Matthews-Cook Y, Kusek J, Nyberg L Jr. The diagnosis of interstitial cystitis revisited: lessons learned from the National Institutes of Health Interstitial Cystitis Database study. *J. Urol.* 1999; **161**: 553–7.

6 Ito T, Miki M, Yamada T. Interstitial cystitis in Japan. *BJU Int.* 2000; **86**: 634–7.

7 Yoshimura N, Seki S, Chancellor MB, de Groat WC, Ueda T. Targeting afferent hyperexcitability for therapy of the painful bladder syndrome. *Urology* 2002; **59** (5 Suppl 1): 61–7.

8 Theoharides TC, Kempuraj D, Sant GR. Mast cell involvement in interstitial cystitis: a review of human and experimental evidence. *Urology* 2001; **57** (6 Suppl 1): 47–55.

9 Sukiennik A, Carr DB, Bonney I, Marchand JE, Wurm H, Sant GR. The effect of short-term epidural local anesthetic blockade on urinary levels of substance P in interstitial cystitis. *Anesth. Analg.* 2004; **98**: 846–50.

10 Lazzeri M, Beneforti P, Benaim G, Maggi CA, Lecci A, Turini D. Intravesical capsaicin for treatment of severe bladder pain: a randomized placebo controlled study. *J. Urol.* 1996; **156**: 947–52.

11 Lazzeri M, Beneforti P, Spinelli M, Zanollo A, Barbagli G, Turini D. Intravesical resiniferatoxin for the treatment of hypersensitive disorder: a randomized placebo controlled study. *J. Urol.* 2000; **164** (3 Pt 1): 676–9.

12 Payne CK, Mosbaugh PG, Forrest JB *et al.* Intravesical resiniferatoxin for the treatment of interstitial cystitis: a randomized, double-blind, placebo controlled trial. *J. Urol.* 2005; **173**: 1590–4.

13 Nickel JC, Moldwin R, Lee S, Davis EL, Henry RA, Wyllie MG. Intravesical alkalinized lidocaine (PSD597) offers sustained relief from symptoms of interstitial cystitis and painful bladder syndrome. *BJU Int.* 2009; **103**: 910–18.

14 Dmitrieva N, McMahon SB. Sensitisation of visceral afferents by nerve growth factor in the adult rat. *Pain* 1996; **66**: 87–97.

15 Dmitrieva N, Shelton D, Rice AS, McMahon SB. The role of nerve growth factor in a model of visceral inflammation. *Neuroscience* 1997; **78**: 449–59.

16 Habler HJ, Janig W, Koltzenburg M. Activation of unmyelinated afferent fibres by mechanical stimuli and inflammation of the urinary bladder in the cat. *J. Physiol.* 1990; **425**: 545–62.

17 Sengupta JN, Gebhart GF. Mechanosensitive properties of pelvic nerve afferent fibers innervating the urinary bladder of the rat. *J. Neurophysiol.* 1994; **72**: 2420–30.

18 Cardenas CG, Del Mar LP, Cooper BY, Scroggs RS. 5HT4 receptors couple positively to tetrodotoxin-insensitive sodium channels in a subpopulation of capsaicin-sensitive rat sensory neurons. *J. Neurosci.* 1997; **17**: 7181–9.

19 England S, Bevan S, Docherty RJ. PGE2 modulates the tetrodotoxin-resistant sodium current in neonatal rat dorsal root ganglion neurones via the cyclic AMP-protein kinase A cascade. *J. Physiol.* 1996; **495** (Pt 2): 429–40.

20 Gold MS, Dastmalchi S, Levine JD. Co-expression of nociceptor properties in dorsal root ganglion neurons from the adult rat in vitro. *Neuroscience* 1996; **71**: 265–75.

21 Yoshimura N, Bennett NE, Hayashi Y *et al.* Bladder overactivity and hyperexcitability of bladder afferent neurons after intrathecal delivery of nerve growth factor in rats. *J. Neurosci.* 2006; **26**: 10847–55.

22 Vizzard MA, Erdman SL, de Groat WC. Increased expression of neuronal nitric oxide synthase in bladder afferent pathways following chronic bladder irritation. *J. Comp. Neurol.* 1996; **370**: 191–202.

23 Vizzard MA, Boyle MM. Increased expression of growth-associated protein (GAP-43) in lower urinary tract pathways following cyclophosphamide (CYP)-induced cystitis. *Brain Res.* 1999; **844**: 174–87.

24 Vizzard MA. Up-regulation of pituitary adenylate cyclase-activating polypeptide in urinary bladder pathways after chronic cystitis. *J. Comp. Neurol.* 2000; **420**: 335–48.

25 Vizzard MA. Alterations in neuropeptide expression in lumbosacral bladder pathways following chronic cystitis. *J. Chem. Neuroanat.* 2001; **21**: 125–38.

26 Dattilio A, Vizzard MA. Up-regulation of protease activated receptors in bladder after cyclophosphamide induced cystitis and colocalization with capsaicin receptor (VR1) in bladder nerve fibers. *J. Urol.* 2005; **173**: 635–9.

27 Alagiri M, Chottiner S, Ratner V, Slade D, Hanno PM. Interstitial cystitis: unexplained associations with other chronic disease and pain syndromes. *Urology* 1997; **49** (5A Suppl): 52–7.

28 Ustinova EE, Fraser MO, Pezzone MA. Colonic irritation in the rat sensitizes urinary bladder afferents to mechanical and chemical stimuli: an afferent origin of pelvic organ cross-sensitization. *Am. J. Physiol. Renal Physiol.* 2006; **290**: F1478–87.

29 Pezzone MA, Liang R, Fraser MO. A model of neural cross-talk and irritation in the pelvis: implications for the overlap of chronic pelvic pain disorders. *Gastroenterology* 2005; **128**: 1953–64.

30 Fitzgerald JJ, Ustinova E, Koronowski KB, de Groat WC, Pezzone MA. Evidence for the role of mast cells in colon-bladder cross organ sensitization. *Auton. Neurosci.* 2013; **173**: 6–13.

31 Pan XQ, Gonzalez JA, Chang S, Chacko S, Wein AJ, Malykhina AP. Experimental colitis triggers the release of substance P and calcitonin gene-related peptide in the urinary bladder via TRPV1 signaling pathways. *Exp. Neurol.* 2010; **225**: 262–73.

32 Malykhina AP, Qin C, Greenwood-van Meerveld B, Foreman RD, Lupu F, Akbarali HI. Hyperexcitability of convergent colon and bladder dorsal root ganglion neurons after colonic inflammation: mechanism for pelvic organ cross-talk. *Neurogastroenterol. Motil.* 2006; **18**: 936–48.

33 Christianson JA, Liang R, Ustinova EE, Davis BM, Fraser MO, Pezzone MA. Convergence of bladder and colon sensory innervation occurs at the primary afferent level. *Pain* 2007; **128**: 235–43.

34 Freund-Michel V, Frossard N. The nerve growth factor and its receptors in airway inflammatory diseases. *Pharmacol. Ther.* 2008; **117**: 52–76.

35 Levi-Montalcini R, Skaper SD, Dal Toso R, Petrelli L, Leon A. Nerve growth factor: from neurotrophin to neurokine. *Trends Neurosci.* 1996; **19**: 514–20.

36 Okragly AJ, Niles AL, Saban R *et al.* Elevated tryptase, nerve growth factor, neurotrophin-3 and glial cell line-derived neurotrophic factor levels in the urine of interstitial cystitis and bladder cancer patients. *J. Urol.* 1999; **161**: 438–41; discussion 441–2.

37 Lowe EM, Anand P, Terenghi G, Williams-Chestnut RE, Sinicropi DV, Osborne JL. Increased nerve growth factor levels in the urinary bladder of women with idiopathic sensory urgency and interstitial cystitis. *Br. J. Urol.* 1997; **79**: 572–7.

38 Yoshimura N, de Groat WC. Increased excitability of afferent neurons innervating rat urinary bladder after chronic bladder inflammation. *J. Neurosci.* 1999; **19**: 4644–53.

39 Evans RJ, Moldwin RM, Cossons N, Darekar A, Mills IW, Scholfield D. Proof of concept trial of tanezumab for the treatment of symptoms associated with interstitial cystitis. *J. Urol.* 2011; **185**: 1716–21.

40 Qiao LY, Vizzard MA. Up-regulation of phosphorylated CREB but not c-Jun in bladder afferent neurons in dorsal root ganglia after cystitis. *J. Comp. Neurol.* 2004; **469**: 262–74.

41 Chuang YC, Fraser MO, Yu Y, Chancellor MB, de Groat WC, Yoshimura N. The role of bladder afferent pathways in bladder hyperactivity induced by the intravesical administration of nerve growth factor. *J. Urol.* 2001; **165**: 975–9.

42 Seki S, Sasaki K, Fraser MO *et al.* Immunoneutralization of nerve growth factor in lumbosacral spinal cord reduces bladder hyperreflexia in spinal cord injured rats. *J. Urol.* 2002; **168**: 2269–74.

43 Oddiah D, Anand P, McMahon SB, Rattray M. Rapid increase of NGF, BDNF and NT-3 mRNAs in inflamed bladder. *Neuroreport* 1998; **9**: 1455–8.

44 Jaggar SI, Scott HC, Rice AS. Inflammation of the rat urinary bladder is associated with a referred thermal hyperalgesia which is nerve growth factor dependent. *Br. J. Anaesth.* 1999; **83**: 442–8.

45 Qiao LY, Vizzard MA. Cystitis-induced upregulation of tyrosine kinase (TrkA, TrkB) receptor expression and phosphorylation in rat micturition pathways. *J. Comp. Neurol.* 2002; **454**: 200–11.

46 Murray E, Malley SE, Qiao LY, Hu VY, Vizzard MA. Cyclophosphamide induced cystitis alters neurotrophin and receptor tyrosine kinase expression in pelvic ganglia and bladder. *J. Urol.* 2004; **172** (6 Pt 1): 2434–9.

47 Klinger MB, Girard B, Vizzard MA. p75NTR expression in rat urinary bladder sensory neurons and spinal cord with cyclophosphamide-induced cystitis. *J. Comp. Neurol.* 2008; **507**: 1379–92.

48 Wakabayashi Y, Maeda T, Kwok YN. Increase of p75 immunoreactivity in rat urinary bladder following inflammation. *Neuroreport* 1996; **7**: 1141–4.

49 Guerios SD, Wang ZY, Boldon K, Bushman W, Bjorling DE. Blockade of NGF and trk receptors inhibits increased peripheral mechanical sensitivity accompanying cystitis in rats. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* 2008; **295**: R111–22.

50 Guerios SD, Wang ZY, Bjorling DE. Nerve growth factor mediates peripheral mechanical hypersensitivity that accompanies experimental cystitis in mice. *Neurosci. Lett.* 2006; **392**: 193–7.

51 Kashyap M, Kawamorita N, Tyagi V *et al.* Down-regulation of nerve growth factor expression in the bladder by antisense oligonucleotides as new treatment for overactive bladder. *J. Urol.* 2013; **190**: 757–64.

52 Yoshimura N, Miyazato M, Kitta T, Yoshikawa S. Central nervous targets for the treatment of bladder dysfunction. *Neurourol. Urodyn.* 2013; **33**: 59–66.

53 Ratner V. Interstitial cystitis: a chronic inflammatory bladder condition. *World J. Urol.* 2001; **19**: 157–9.

54 Glazer EJ, Basbaum AI. Leucine enkephalin: localization in and axoplasmic transport by sacral parasympathetic preganglionic neurons. *Science* 1980; **208**: 1479–81.

55 Dray A, Nunan L, Wire W. Central delta-opioid receptor interactions and the inhibition of reflex urinary bladder contractions in the rat. *Br. J. Pharmacol.* 1985; **85**: 717–26.

56 Yokoyama H, Sasaki K, Franks ME *et al.* Gene therapy for bladder overactivity and nociception with herpes simplex virus vectors expressing preproenkephalin. *Hum. Gene Ther.* 2009; **20**: 63–71.

57 Yokoyama H, Oguchi T, Goins WF *et al.* Effects of herpes simplex virus vector-mediated enkephalin gene therapy on bladder overactivity and nociception. *Hum. Gene Ther.* 2013; **24**: 170–80.

58 Chuang YC, Chou AK, Wu PC *et al.* Gene therapy for bladder pain with gene gun particle encoding pro-opiomelanocortin cDNA. *J. Urol.* 2003; **170**: 2044–8.

59 Lu CY, Chou AK, Wu CL *et al.* Gene-gun particle with pro-opiomelanocortin cDNA produces analgesia against formalin-induced pain in rats. *Gene Ther.* 2002; **9**: 1008–14.

60 Mousa SA, Zhang Q, Sitte N, Ji R, Stein C. beta-Endorphin-containing memory-cells and mu-opioid receptors undergo transport to peripheral inflamed tissue. *J. Neuroimmunol.* 2001; **115**: 71–8.

61 Sawynok J, Liu XJ. Adenosine in the spinal cord and periphery: release and regulation of pain. *Prog. Neurobiol.* 2003; **69**: 313–40.

62 Godfrey L, Yan L, Clarke GD, Ledent C, Kitchen I, Hourani SM. Modulation of paracetamol antinociception by caffeine and by selective adenosine A2 receptor antagonists in mice. *Eur. J. Pharmacol.* 2006; **531**: 80–6.

63 Hussey MJ, Clarke GD, Ledent C, Hourani SM, Kitchen I. Reduced response to the formalin test and lowered spinal NMDA glutamate receptor binding in adenosine A2A receptor knockout mice. *Pain* 2007; **129**: 287–94.

64 Zeilhofer HU. The glycinergic control of spinal pain processing. *Cell. Mol. Life Sci.* 2005; **62**: 2027–35.

65 Miyazato M, Sugaya K, Nishijima S, Kadekawa K, Ashimine S, Ogawa Y. Intrathecal or dietary glycine inhibits bladder and urethral activity in rats with spinal cord injury. *J. Urol.* 2005; **174**: 2397–400.

66 Miyazato M, Sugaya K, Nishijima S *et al.* Changes of bladder activity and glycine levels in the lumbosacral cord after partial bladder outlet obstruction in rats. *Int. J. Urol.* 2008; **15**: 843–7.

67 Nishijima S, Sugaya K, Fukuda T, Miyazato M, Ashimine S, Ogawa Y. Serum amino acids as indicators of cerebrospinal neuronal activity in patients with micturition disorders. *Int. J. Urol.* 2006; **13**: 1479–83.

68 Zafra F, Aragon C, Olivares L, Danbolt NC, Gimenez C, Storm-Mathisen J. Glycine transporters are differentially expressed among CNS cells. *J. Neurosci.* 1995; **15** (5 Pt 2): 3952–69.

69 Eulenburg V, Armsen W, Betz H, Gomeza J. Glycine transporters: essential regulators of neurotransmission. *Trends Biochem. Sci.* 2005; **30**: 325–33.

70 Zafra F, Gomeza J, Olivares L, Aragon C, Gimenez C. Regional distribution and developmental variation of the glycine transporters GLYT1 and GLYT2 in the rat CNS. *Eur. J. Neurosci.* 1995; **7**: 1342–52.

71 Dohi T, Morita K, Kitayama T, Motoyama N, Morioka N. Glycine transporter inhibitors as a novel drug discovery strategy for neuropathic pain. *Pharmacol. Ther.* 2009; **123**: 54–79.

72 Haranishi Y, Hara K, Terada T, Nakamura S, Sata T. The antinociceptive effect of intrathecal administration of glycine transporter-2 inhibitor ALX1393 in a rat acute pain model. *Anesth. Analg.* 2010; **110**: 615–21.

73 Yoshikawa S, Oguchi T, Funahashi Y, de Groat WC, Yoshimura N. Glycine transporter type 2 (GlyT2) inhibitor ameliorates bladder overactivity and nociceptive behavior in rats. *Eur. Urol.* 2012; **62**: 704–12.

74 Erickson DR, Xie SX, Bhavanandan VP *et al*. A comparison of multiple urine markers for interstitial cystitis. *J. Urol.* 2002; **167**: 2461–9.

75 Keay S. Cell signaling in interstitial cystitis/painful bladder syndrome. *Cell. Signal.* 2008; **20**: 2174–9.

76 Ogawa T, Homma T, Igawa Y *et al*. CXCR3 binding chemokine and TNFSF14 over expression in bladder urothelium of patients with ulcerative interstitial cystitis. *J. Urol.* 2010; **183**: 1206–12.

77 Peters KM, Diokno AC, Steinert BW. Preliminary study on urinary cytokine levels in interstitial cystitis: does intravesical bacille Calmette-Guerin treat interstitial cystitis by altering the immune profile in the bladder? *Urology* 1999; **54**: 450–3.

78 Gautam S, Tebo JM, Hamilton TA. IL-4 suppresses cytokine gene expression induced by IFN-gamma and/or IL-2 in murine peritoneal macrophages. *J. Immunol.* 1992; **148**: 1725–30.

79 te Velde AA, Huijbens RJ, Heije K, de Vries JE, Figdor CG. Interleukin-4 (IL-4) inhibits secretion of IL-1 beta, tumor necrosis factor alpha, and IL-6 by human monocytes. *Blood* 1990; **76**: 1392–7.

80 Ueda T, Tamaki M, Ogawa O, Yamauchi T, Yoshimura N. Improvement of interstitial cystitis symptoms and problems that developed during treatment with oral IPD-1151T. *J. Urol.* 2000; **164**: 1917–20.

81 Rotondi M, Chiovato L, Romagnani S, Serio M, Romagnani P. Role of chemokines in endocrine autoimmune diseases. *Endocr. Rev.* 2007; **28**: 492–520.

82 Bonecchi R, Bianchi G, Bordignon PP *et al*. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. *J. Exp. Med.* 1998; **187**: 129–34.

83 Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. *J. Exp. Med.* 1998; **187**: 875–83.

84 Sakthivel SK, Singh UP, Singh S, Taub DD, Novakovic KR, Lillard JW Jr. CXCL10 blockade protects mice from cyclophosphamide-induced cystitis. *J. Immune Based Ther. Vaccines* 2008; **6**: 6.

85 van de Merwe JP, Nordling J, Bouchelouche P *et al*. Diagnostic criteria, classification, and nomenclature for painful bladder syndrome/interstitial cystitis: an ESSIC proposal. *Eur. Urol.* 2008; **53**: 60–7.

86 Corcoran AT, Yoshimura N, Tyagi V, Jacobs B, Leng W, Tyagi P. Mapping the cytokine profile of painful bladder syndrome/interstitial cystitis in human bladder and urine specimens. *World J. Urol.* 2013; **31**: 241–6.

87 Keystone EC, Ware CF. Tumor necrosis factor and anti-tumor necrosis factor therapies. *J. Rheumatol. Suppl.* 2010; **85**: 27–39.

88 Marchand F, Perretti M, McMahon SB. Role of the immune system in chronic pain. *Nat. Rev. Neurosci.* 2005; **6**: 521–32.

89 Schafers M, Svensson CI, Sommer C, Sorkin LS. Tumor necrosis factor-alpha induces mechanical allodynia after spinal nerve ligation by activation of p38 MAPK in primary sensory neurons. *J. Neurosci.* 2003; **23**: 2517–21.

90 Sommer C, Lindenlaub T, Teuteberg P, Schafers M, Hartung T, Toyka KV. Anti-TNF-neutralizing antibodies reduce pain-related behavior in two different mouse models of painful mononeuropathy. *Brain Res.* 2001; **913**: 86–9.

91 Sommer C, Schmidt C, George A. Hyperalgesia in experimental neuropathy is dependent on the TNF receptor 1. *Exp. Neurol.* 1998; **151**: 138–42.

92 Funahashi Y, Oguchi T, Goins WF *et al*. Herpes simplex virus vector mediated gene therapy of tumor necrosis factor-alpha blockade for bladder overactivity and nociception in rats. *J. Urol.* 2013; **189**: 366–73.

93 Hao S, Mata M, Glorioso JC, Fink DJ. HSV-mediated expression of interleukin-4 in dorsal root ganglion neurons reduces neuropathic pain. *Mol. Pain* 2006; **2**: 6.

94 Oguchi T, Funahashi Y, Yokoyama H *et al*. Effect of herpes simplex virus vector-mediated interleukin-4 gene therapy on bladder overactivity and nociception. *Gene Ther.* 2013; **20**: 194–200.

Review Article

Intravesical Liposome and Antisense Treatment for Detrusor Overactivity and Interstitial Cystitis/Painful Bladder Syndrome

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Purpose. The following review focuses on the recent advancements in intravesical drug delivery, which brings added benefit to the therapy of detrusor overactivity and interstitial cystitis/painful bladder syndrome (IC/PBS). *Results*. Intravesical route is a preferred route of administration for restricting the action of extremely potent drugs like DMSO for patients of interstitial cystitis/painful bladder syndrome (IC/PBS) and botulinum toxin for detrusor overactivity. Patients who are either refractory to oral treatment or need to mitigate the adverse effects encountered with conventional routes of administration also chose this route. Its usefulness in some cases can be limited by vehicle (carrier) toxicity or short duration of action. Efforts have been underway to overcome these limitations by developing liposome platform for intravesical delivery of biotechnological products including antisense oligonucleotides. *Conclusions*. Adoption of forward-thinking approaches can achieve advancements in drug delivery systems targeted to future improvement in pharmacotherapy of bladder diseases. Latest developments in the field of nanotechnology can bring this mode of therapy from second line of treatment for refractory cases to the forefront of disease management.

1. Introduction

Intravesical therapies have demonstrated varying degrees of efficacy and safety in treatment of interstitial cystitis/painful bladder syndrome (IC/PBS) [1] and overactive bladder OAB [2]. Pharmacotherapy by this route provides high local drug concentrations in the bladder with low risk of systemic side effects [3]. Conventional therapies for OAB and detrusor overactivity (DO), either neurogenic or idiopathic, have limited efficacy and acceptability. Anticholinergic medications, which are currently the mainstay of the treatment of OAB, are not always effective and often have undesirable side effects such as dry mouth and constipation [4]. Therefore, search for alternative therapies directed against local targets with fewer side effects is encouraged.

The IC/PBS syndrome is characterized by pelvic pain and urinary storage symptoms (e.g., urinary urgency and frequency). The O'Leary-Sant symptom and problem score (interstitial cystitis symptom index (ICSI) and problem index (ICPI)) is recognized as one of the most reliable and valid instruments to identify the extent of bothersome symptoms and the most prominent voiding and painful symptoms in IC/PBS patients [5, 6]. Pentosan polysulfate, PPS, is a synthetic sulphated polysaccharide and is the only approved oral drug for IC/PBS, but it requires daily administration for 6 months before any benefit accrues for the patients [7, 8]. Less than one-tenth of oral dose is excreted into the urine of IC/PBS patients, which is considered to replenish the damaged glycosaminoglycan GAG layer and reduce the influx of potassium back into bladder from urine.

Orally administered agents are often unable to create effective luminal drug concentration due to low urinary excretion of drugs, which justifies bladder instillation. Replacement of GAG layer by intravesical administration of hyaluronic acid has been successfully tried in IC/PBS patients [9]. Intravesical therapy also holds the potential to facilitate

the separation of therapeutic actions from side effects by involving a diverse array of novel chemical, pharmacological, and formulation strategies. However, drug delivery by intravesical route is constrained by the impermeability of the urothelium, short duration of action, and the need for frequent administration.

The urinary bladder lining is the most impermeable barrier in the human body [10, 11]. Therefore, therapies delivered directly to the bladder lumen have limited opportunity for systemic distribution, which typically leads to fewer side effects. In addition, the mechanisms for locally delivered therapies can be independent of existing oral therapies, and in some cases, an additive effect can provide compelling comarketing opportunities [12]. We will review the current understanding of urothelium structure and role of intravesical drug delivery in unmasking the pharmacological function of different receptors expressed on its luminal surface.

2. Urothelium

Recent investigations have revealed that the urothelium is not just a physical barrier between blood and urine but can express a host of receptors having a functional significance in micturition reflex. The recent identification of a cannabinoid, nicotinic, neurokinin receptors and potassium ion channels in urothelium [13–16] have revealed the role of urothelium as an excitable cell layer in bladder that responds to stretch and convey messages to underlying afferents in bladder. There is also mounting evidence to demonstrate expression of adrenergic, bradykinin, and transient receptor potential (TRP) receptors in urothelium and in proximity of afferent nerves [17, 18]. Urothelium is the primary nonneuronal source for the release of molecules such as adenosine triphosphate (ATP), acetylcholine, and nitric oxide, which are known to affect micturition [19, 20]. Intravesical therapy can be used to unravel the pharmacology of these receptors and paracrine messengers released from urothelium [21, 22].

3. Muscarinic Receptors

It is widely accepted that oral antimuscarinics act on muscarinic receptors in the detrusor for managing the symptoms of DO and OAB. Conventional wisdom largely ignores the role of muscarinic receptors expressed on urothelium [23]. Muscarinic receptors expressed on urothelium are believed to be involved in afferent signaling for micturition [23]. The afferent signals are believed to be generated from the basal nonneuronal acetylcholine released during the storage phase from urothelium to enhance the myogenic contractile activity of the detrusor [19, 20].

Theoretically speaking, not only can receptors expressed on the urothelium be influenced by antimuscarinics via the bloodstream, but also few selected antimuscarinics and their active metabolites can affect the muscarinic receptors from the luminal side following their excretion into urine [12]. Alternative mode of action for two antimuscarinic drugs, trospium and solifenacin, was demonstrated by our group [21, 22]. DO in the rat was mimicked by intravesical carbachol

[21, 22]. Urine collected from human volunteers who took trospium and solifenacin was then instilled into rat bladder to determine the effect of the drug fraction excreted into urine. Therefore, intravesical therapy can assist in elucidating the yet unexplored mechanisms for improvement of OAB symptoms by antimuscarinics.

4. Liposomes

Liposomes were earliest prototype of nanoparticles (particles with one of the dimensions in nanometers) that are described as lipid vesicles composed of concentric phospholipid bilayers enclosing an aqueous interior [24, 25]. The lipid vesicles comprise either one or several aqueous compartments delineated by either one (unilamellar) or several (multilamellar) phospholipid bilayers [26]. Liposomes have been widely studied as drug carriers for a variety of chemotherapeutic agents (approximately 40,000 scientific articles have been published on the liposomes use so far) [25, 27]. Liposomes improved the delivery of chemotherapeutic agents by altering pharmacokinetics and reducing toxicity [26, 28, 29]. The success of liposomes in the clinic has been attributed to the nontoxic nature of the lipids used in their formulation.

5. Empty Liposomes

Empty liposomes itself can act as a topical healing agent and same has been demonstrated in treatment of dry eye [30, 31]. Either empty or with entrapped drugs, liposomes have also been used in ophthalmology to ameliorate keratitis, corneal transplant rejection, uveitis, endophthalmitis, and proliferative vitreoretinopathy [32]. These reports encouraged investigation of empty liposomes as a therapeutic agent for bladder injury. Interaction of liposomes with cultured urothelium cells suggested that liposomes can be adsorbed and be endocytosed [33]. Previous studies showed that binding of large multilamellar liposomes to the bladder cells was stronger than of sonicated small size liposomes [34, 35].

5.1. Preclinical Studies. A rat model of bladder injury induced by protamine sulfate [36] was used to asses efficacy of empty liposomes. Installation of liposomes protected bladder irritation induced by protamine sulfate instillation into rat bladder [37] (Figure 1). In earlier study, empty liposomes were used as controls against capsaicin delivery study, which reported tolerance of empty liposomes in uninjured bladder [38]. Bladder tolerance was investigated by cystometry and histology [38].

Physiological effect of liposomes on bladder irritation model induced by protamine sulfate was studied in separate studies [39, 40]. Cystometric studies involved bladder injury induced by infusion of protamine sulfate for an hour followed by irritation caused by infusion of high concentration of potassium chloride solution [39, 40]. Post-treatment of liposomes demonstrated the protective effect in this model [39, 40], which involved coadministration of liposomes with potassium chloride to mimic the clinical disease condition. The comparative efficacy of liposomes was evaluated against

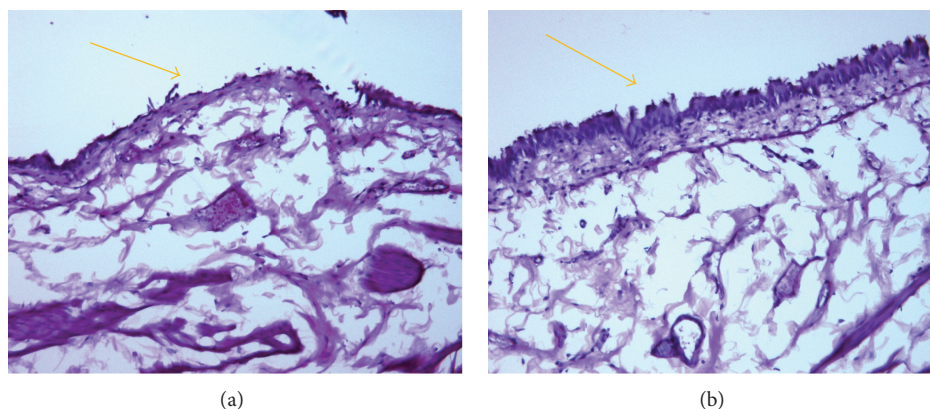(a)   (b)

Figure 1: Protective effect of liposomes evaluated by histology in rat bladder injured by protamine sulfate. Focal loss of apical layer and ulcerated areas were noted in saline treated rats (a). Liposome post-treatment protected rats from ulceration induced by PS as evident from photograph in (b) taken on bladder harvest at 8 h after liposome instillation.

FDA approved therapies of dimethyl sulphoxide (DMSO) and intravesical instillation of PPS [1].

Clinically, DMSO (RIMSO-50) is the only FDA approved intravesical treatment for PBS/IC, [41] but off-label instillation of PPS has also been pursued [42]. The efficacy of various treatments was evaluated in chemically induced bladder hyperactivity in rats by sequential infusion of protamine sulfate and potassium chloride. Bladder reflex activity of female Sprague-Dawley rats before and after treatment was evaluated by continuous cystometry under urethane anaesthesia (1.0 g/kg). Intravesical liposomes were effective in doubling the intercontractile interval (ICI) compared with PPS, while acute instillation of DMSO failed to produce any protective effect in this animal model [43].

Recently, Lee et al. [44] further improved this model of DO induced by intravesical infusion by combining it with systemic metabolic alteration through fructose feeding. Metabolic syndrome created by feeding of fructose to rats can lead to DO and urination frequency [44]. Cystometric bladder capacity of fructose fed rats can be further decreased by instillation of acidic ATP solution, which provokes reflex micturition via afferent noise. Evidence suggests that increased expression or release of neurotransmitters in the mucosal layer of the bladder can generate afferent noise via C-fiber pathway and result in DO [45]. Compared to infusion with normal saline, ATP solution decreased bladder capacity and increased phasic contractions. Addition of liposomes to the ATP solution partially reversed the ATP solution-induced response [46]. Capsaicin induced desensitization also blunted the provocation of ATP in this model to demonstrate the role of afferent noise via C-fibers.

5.2. Clinical Studies. Encouraged by the exciting preclinical efficacy of empty liposomes as a therapeutic agent for intravesical therapy of IC/PBS, Chuang et al. recently published the clinical safety and efficacy of liposomes in IC/PBS patients [47]. In an open label prospective study on 24 IC/PBS patients, the effect of intravesical liposomes was compared against oral PPS. Patients were equally divided into the two treatment arms, administered either intravesical liposomes (80 mg/40 cc distilled water) once weekly or oral PPS (100 mg) 3 times daily for 4 weeks each. Ten possible responses to treatment were monitored at 3 time points, including baseline, and at weeks 4 and 8.

Comparable efficacy of liposomes to oral PPS was demonstrated by statistically significant decreases in urinary frequency and nocturia in both treatment arms. Liposome treated patients showed statistically significant decreases in pain, urgency, and the O'Leary-Sant symptom score, with the effect being most profound on urgency. None of the treated patients in the study reported urinary incontinence, retention, or infection due to liposome instillation and there were no unanticipated adverse events and no significant worsening of symptoms during followup. Intravesical instillation of liposomes in IC/PBS patients was found to be safe with potential improvement after 1 course of therapy for up to 8 weeks. The study design was suboptimal, with lack of blinding and two treatment arms assigned to different routes of drug administration and regimen. Still once weekly liposome therapy was able to demonstrate efficacy comparable to thrice daily oral administration of PPS.

Subsequently, Lee et al. assessed the safety and efficacy of twice weekly administration of liposomes on IC/PBS symptoms [48]. Five patients were given twice a week treatment of liposomes for 4 weeks. The primary outcome was the change in the O'Leary-Sant symptom/problem score and O'Leary-Sant total score from baseline to week 4 and week 8. The O'Leary-Sant symptom/problem score, O'Leary-Sant total score, and pain score at the 4-week followup showed significantly greater improvement from baseline with biweekly instillation than once a week instillation. Tolerability of liposomes remains unchanged from once a week regimen to twice a week regimen. The followup at 8 weeks was also similar for both treatment regimens. The incidence of urinary incontinence, retention, or unanticipated adverse changes was not noted with any regimen. Intravesical liposomes appear to be a promising new treatment for IC/PBS and future large-scale placebo controlled studies are needed to verify these results from a pilot study.
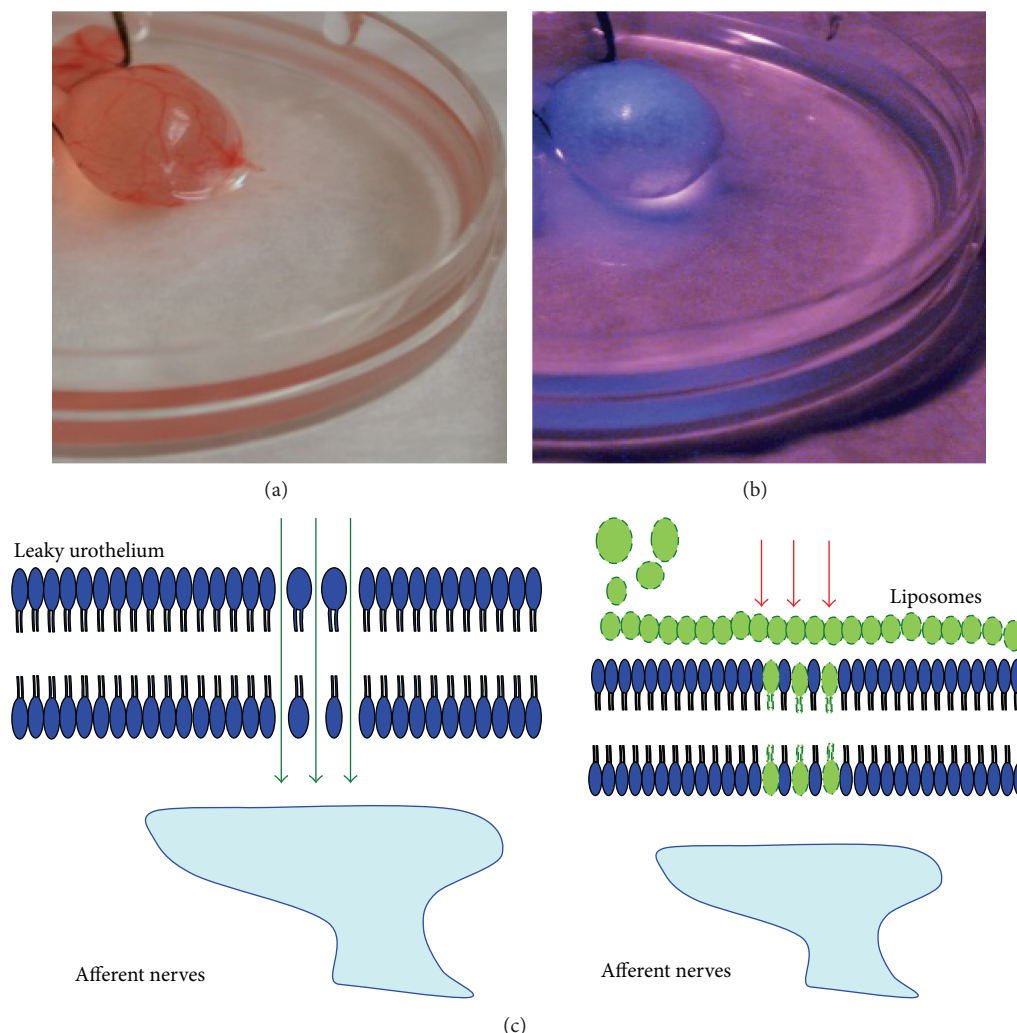
FIGURE 2: *Ex vivo* images of rat bladder taken in visible and near-infrared light indicate the coating formed by instilled liposomes on bladder surface. Liposomes carry a trace amount of near-infrared (NIR) lipophilic carbocyanine dye 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR) that fluoresces on exposure to NIR light. Rat bladder filled with urine was tied at the base with thread prior to harvest. Liposomes coating the bladder surface are invisible in visible light photograph (a) but is indicated by blue colored coating on the bladder luminal surface in NIR light (b). NIR imaging *in vivo* can allow noninvasive repeat objective measurement for bladder residence time of instilled treatments. (c) Schematic illustration of liposome coating the bladder surface. Given the chemical affinity of phospholipids of instilled liposomes with lipids in cells lining the bladder surface, the liposomes form a protective film coating on the injured bladder lumen surface and assist in the repair of leaky and inflamed uroepithelium.

The exact mechanism of action for liposomes in IC/PBS remains to be established, but protective coating effect based on preclinical studies cannot be ruled out as illustrated in Figure 2.

6. Liposomes as a Delivery Platform

Not only is the lining of the urinary bladder the most impermeable tissue in the human body, but it is also the most compliant. As a bladder lining expands, additional membrane material is added to its cells to help retain impermeability [49]. Therefore, vesicular trafficking may provide a favorable environment for drug delivery and therefore it is worth investigating whether vesicle nature of liposomes can aid in improving the delivery of cargo across the bladder permeability barrier.

In the field of neurourology, instillation of neurotoxins into bladder is an accepted approach to achieve chemical neuromodulation of afferent neurotransmission underlying neurogenic bladder and IC/PBS [50]. Existing approaches of chemical neuromodulation by intravesical neurotoxins are suboptimal due to vehicle toxicity for capsaicin [51] or degradation of botulinum toxin (BoNT) in urine. Possible reasons underlying the lack of efficacy from BoNT instillation in bladder can be protein degradation by proteases and proteinases in urine, dilution in urine, or poor uptake of the BoNT solution into the urothelium.

Liposomes have been previously studied as a carrier of toxins to enhance efficacy at lower doses [52]. In the context of
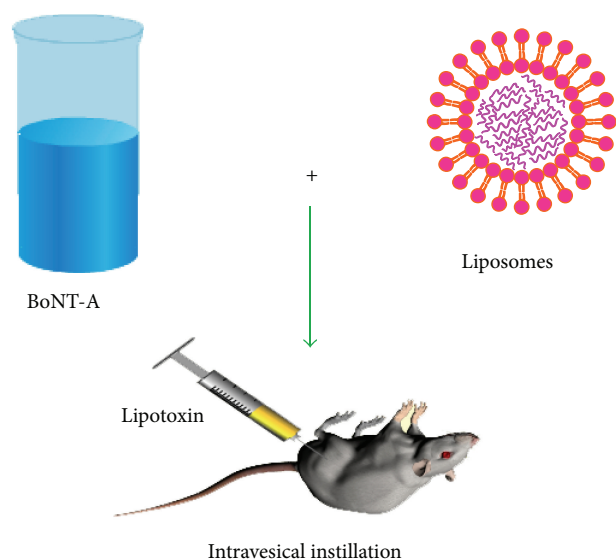
FIGURE 3: Scheme for formulation of BoNT into liposomes for bladder instillation. Freeze dried BoNT is resuspended in distilled water and added to vials containing a desiccated liposomal preparation made of phospholipids. This reconstituted suspension is allowed to hydrate at room temperature for 30–60 min before instillation.

toxins instilled in the bladder, fat-soluble neurotoxin such as capsaicin can be integrated into the phospholipid bilayer [38] and water-soluble neurotoxin such as BoNT can be protected inside the aqueous compartment(s) of liposomes delimited by the phospholipid bilayer(s) [53] (Figure 3). Cystoscope guided injections are the current standard practice in the clinic for administering BoNT to the bladder. But in recent years, studies have also assessed the potential for intravesical instillation of BoNT alone in animal models of bladder irritation [54].

Development of instillation as a mode for administering BoNT in patients will drastically bring down the cost of treatment for patients with refractory overactive bladder. Other groups have recently reported the use of DMSO for instillation of BoNT instead of injection [55]. DMSO does not afford the natural state of the BoNT as a protein and need to be formulated immediately before instillation. Dissolving BoNT in DMSO may not be advisable owing to concerns of BoNT uptake into the systemic circulation of patient. Moreover, biochemical studies have demonstrated that metalloproteolytic activity of the BoNT is strongly enhanced by the presence of lipid membranes [56].

Recent studies reaffirmed the potential of liposomes as a promising vehicle for delivery of neurotoxins to the bladder [38, 53]. The transport of BoNT into urothelium from liposomes was confirmed by detection of its unique effect on neurotransmitters and proteolysis of synaptosomal-associated protein SNAP-25 through immunohistochemistry [53]. The protection of BoNT entrapped inside liposomes from degradation in urine without compromising efficacy was demonstrated by attenuation of acetic acid induced bladder irritation in rats [53]. Similar results were obtained in preclinical studies with liposomes encapsulating capsaicin [38].

Liposomes have proven themselves as biocompatible delivery agents in the bladder.

7. Intravesical Antisense Therapeutics

The term "antisense" therapeutics emerged from seminal studies done 4 decades using a short synthetic oligonucleotide for sequence-specific gene silencing [57]. Gene silencing involves introduction of short strands of DNA (termed as antisense) with sequences complementary to the mRNA encoding a particular gene inside the cell with the intent to block gene expression through either translational inhibition or enzymatic cleavage of the mRNA target [3].

Oligonucleotide ODN binds specifically and strongly to the mRNA target through Watson-Crick base pairing. ODN can be basically categorized into those that direct cleavage of the target mRNA as caused by small interfering RNAs (siRNAs) and those that alter mRNA translation without causing mRNA cleavage. Recent discovery of small interfering RNAs (siRNAs) and the elucidation of the RNA interference (RNAi) pathway has also brought a sea change in the control of posttranscriptional gene expression. siRNA takes advantage of endogenous cellular pathways to potently silence the expression of specific genes (Figure 4).

Antisense mechanism is a promising approach for developing therapeutics based on rational gene-based drug design. Antisense therapeutics have been under clinical investigation for more than 30 years [58]. However, drug development of this approach has been hampered by inefficient intracellular delivery and cellular uptake of the ODN. The translation of basic antisense research into therapeutics is also impeded by intracellular stability of ODN and potential for "off-target" gene silencing, immunostimulation, and other side effects.

A vast array of chemical modifications to ODN has been developed to overcome the therapeutically limiting features by altering internucleotide phosphate linkages, backbone sugars, or nucleobases. One such modified ODN is peptide nucleic acid, which replaces phosphodiester bond between nucleobases with a peptide bond. Replacement of phosphodiester bond by a phosphorothioate linkage is another method to improve the stability of ODN against nucleases.

Great progress has been made in translating antisense research into clinical therapies based on local injection into eye [58]. The field recently progressed further with a systemic injection therapy for treating familial hypercholesterolemia [59]. Antisense therapeutics have been used for exon skipping to optimize the functionality of a truncated dystrophin protein in dog model of Duchenne muscular dystrophy [60].

Nevertheless, applied research for bladder diseases has lagged behind, considering that the anatomical architecture of bladder provides ease of local administration with restricted systemic side effects due to lower serum uptake of antisense ODN. Bladder instillation of antisense ODN or their chemically modified mimics can therefore be an efficient means to control the expression of therapeutically relevant genes. Antisense agents can also be used to elucidate the role of newly discovered genes in bladder function.
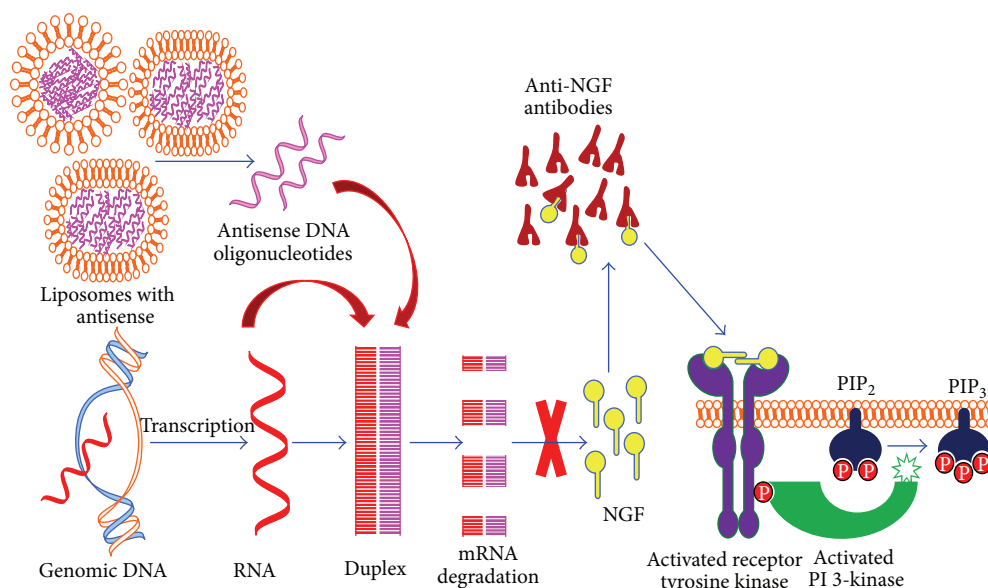
FIGURE 4: Illustration of mechanisms for blocking NGF expression by either NGF antibodies or downregulation of NGF mRNA expression. NGF antibody sequesters the freely available NGF and prevents it from binding with the cognate TrkA receptors. NGF antisense selectively binds with NGF mRNA inside the cells to block the translation of corresponding mRNA. siRNA takes advantage of endogenous cellular pathways to degrade NGF mRNA and control the posttranscriptional NGF gene expression.

8. NGF Expression in Bladder

Several studies have reported that patients with OAB or IC/PBS excrete more nerve growth factor (NGF) in their urine relative to asymptomatic controls [61]. Higher expression of NGF in bladder of patients [62], with corresponding lower serum levels of NGF, makes the bladder tissue the likely source for the elevated NGF in urine [63]. Previous studies have indicated that increased levels of NGF in the bladder and bladder afferent pathways are directly involved in the emergence of hyperexcitability of C-fiber bladder sensory pathways leading to the pathology of DO and OAB (Figure 4) [64].

In addition, intrathecal application of NGF antibodies reduced NGF levels in bladder afferent pathways and normalized bladder/urethral function in spinal cord injured (SCI) rats [65]. Because it is likely that the major pathology of OAB is driven by NGF, targeting the intracellular synthesis of NGF molecule in bladder is a promising therapeutic alternative.

9. NGF Expression a Drug Target

Overexpression of NGF can be blocked either directly by antibodies [66] or by blocking the synthesis of NGF protein from mRNA [67]. Systemic administration of monoclonal human NGF antibodies (tanezumab) has been explored for therapeutic outcomes in IC/PBS patients but not without encountering safety concerns such as paresthesia, hypoesthesia, and arthralgia [66]. Generalized blockade of NGF activity at sites other than bladder by anti-NGF antibodies may not be the preferred outcome, because NGF is an essential housekeeping growth factor necessary for the survival and growth of neurons [68]. The physiological necessity of NGF

action at those sites may explain the incidence of paresthesia, hypoesthesia, and arthralgia in patients treated with systemic anti-NGF antibodies [66].

9.1. Peptide Nucleic Acid (PNA). Therefore, to reduce the toxicity of systemic blockade of NGF, we sought to develop a novel intravesical therapy of OAB by targeting the intracellular synthesis of NGF in the urothelium. Antisense ODN needed to be able to cross cell membrane to act as a drug and negatively charged ODN will not pass through a lipid layer such as cell membranes. Bladder uptake of ODN is limited by the anionic charge and size of the ODN as well as anionic glycosaminoglycan layer of the inner bladder surface. Therefore, primary impediment to be overcome in the development of intravesical antisense therapeutics is inefficient bladder uptake of the ODN across urothelium. Previous studies showed that heparan sulfate proteoglycans expressed on cell surface act as receptors for extracellular TAT uptake [69]. Therefore, it was reasoned that GAG layer on bladder surface can facilitate bladder uptake of peptide nucleic acid PNA, if it could be conjugated with synthetic TAT peptide.

Water insoluble peptide nucleic acid targeting NGF was conjugated with cell penetrating cationic peptide TAT for intracellular delivery of antisense moiety to demonstrate efficacy in animal models. Studies showed that PNA conjugated with TAT suppressed cyclophosphamide cystitis following local instillation of antisense against NGF [67]. There was negligible uptake of peptide nucleic acid in absence of TAT conjugation. Prior to determination of *in vivo* efficacy of conjugate, suitable target sequence on NGF mRNA was determined by predicted folding structure and cell transfection experiments. Successful intravesical delivery of peptide

nucleic acid in bladder at the same time of cyclophosphamide injection protected against the cystitis by blocking the rise in bladder contraction frequency and inflammation.

9.2. Phosphorothioate-Linked Analogues.

Considering the difficulty and possible nonspecific toxicity of peptide nucleic acid and TAT, recent developments focused on simplifying the approach using water-soluble phosphorothioated ODN, which have an increased resistance to exo- and endonucleases for improved stability [70]. As alluded to in the above text, success of antisense therapeutics is largely dependent on the development of a delivery vehicle that can efficiently deliver antisense ODN in bladder. Preliminary studies showed that liposomes can be far better biocompatible effective carriers for local gene silencing of NGF gene in bladder cells.

Cationic liposomes and mimics have emerged as the most popular nonviral method to deliver nucleic acids in therapeutic applications. Easy and reversible complex formation of cationic liposomes with ODN at room temperature allows their use as carriers. Electrostatic attraction between the cationic lipid, DOTAP, and the polyanionic antisense ODN is responsible for the complex formation. The efficacy of liposome delivered siRNA by intravesical route has been previously demonstrated in preclinical models of bladder cancer [71]. Residence of ODN in the rat bladder after intravesical instillation was demonstrated to be longer than 24 h using fluorescent tagged ODN complexed with liposomes [72]. Fluorescent probe was localized in bladder urothelium cells 24 h following instillation. The 24 h residence time was also demonstrated for siRNA in mouse bladder [73]. Bladder uptake of fluorescent ODN without liposomes in normal rat bladder is poor. Previous studies have shown that very high concentration of phosphorothioated ODN can deliver ODN without liposomes to bladder cells of mice having bladder cancer [74]. The urothelium barrier may be slightly compromised in cancerous condition and the strategy of loading bladder with high dose of ODN may not work in noncancerous diseased condition of bladder with intact barrier.

In order to evaluate the efficacy of NGF antisense ODN, acetic acid infusion was used to cause a rapid rise of NGF protein levels. A single dose instillation of OND complexed with liposomes protected against bladder overactivity (BO) induced by acetic acid. Together with data of bladder uptake studies using fluorescent ODN, it is demonstrated that OND is readily available to the bladder after intravesical instillation. It has been previously reported that, within 2 h of exposure to irritants such as turpentine and acetic acid, there occurs a rapid rise in bladder content of NGF [75]. Later studies found that bladder responds to insults with upregulation in the genes for NGF, sE-Selectin and receptor for monocyte chemoattractant protein-1 (MCP-1) within 30 min of exposure to lipopolysaccharide (Figure 5) [76]. The acetic acid induced overexpression of NGF was blunted by pretreatment with NGF antisense OND with phosphorothioate linkage prior to exposure of acetic acid. The downregulation of NGF mRNA expression was in agreement with reduced protein levels and suppressed NGF-like immunoreactivity in the urothelium.

9.3. Antisense and Downstream Signalling of NGF.

Drug development of intravesical antisense for NGF was also able to unmask the downstream signaling [77] involving NGF following exposure to acetic acid. Experiments supported the role of NGF as a paracrine messenger [77], which is known to activate several downstream effectors to manifest physiological and pathological signaling changes linked to it [76, 78–80]. The bladder injury set off by acetic acid initiates the signalling cascades that upregulate the expression of NGF and other chemokines, MCP-1, CXCL-1, and CXCL-10, and prostaglandins [76, 78–80]. As reported elsewhere, localization of chemokines within synaptic vesicles in neurons [81] is consistent with their ability to act as excitatory neurotransmitters following AA exposure.

Chemokines are one of several downstream effectors activated by NGF [76, 78–80] and interestingly chemokine receptors are widely expressed in neural and nonneural elements of the nociceptive pathways that are responsible for visceral and somatic pain sensation [82]. MCP-1 [83] and CXCL-10 [84] are constitutively expressed in neurons, where they participate in excitability of primary afferent neurons through transactivation of transient receptor channels and nociceptor sensitization [84]. Overexpression of NGF is likely to drive the expression of VEGF from neurons and leptin [78] from adipocytes covering neurons [85].

Cooperative expression of NGF and MCP-1 is able to induce hyperexcitability in neurons by activating TRPV1 receptor. In addition, MCP-1, CXCL-1, and CXCL-10 cause chemoattraction of monocytes, neutrophils, and lymphocytes, respectively, to mediate the bladder injury set off by acetic acid. Extravasation is an essential prerequisite for infiltration of monocytes, neutrophils, and lymphocytes, which requires the expression of adhesion molecules like E-selectin and intracellular adhesion molecule ICAM-1 [76, 86]. E-selectin is a cytokine-inducible adhesion molecule that supports the rolling and stable arrest of leukocytes on activated vascular endothelium. Expression of E-selectin has been linked to adherence of neutrophils to bladder microvascular endothelial cells and to cyclophosphamide cystitis [87, 88]. E-selectin gene expression is activated by the NF-κB and MAP kinase signal transduction pathways [76, 86].

Activation and recruitment of leukocytes to acetic acid induced bladder injury also require expression of intracellular adhesion molecule ICAM-1 [89]. ICAM-1 interacts specifically with its receptors of the integrin family to induce reversible cell-cell interactions involving adhesion. Expression of ICAM-1 was found to be increased in patients with IC/PBS and reduced in patients responding to instillation of hyaluronic acid [90]. Expression of ICAM-1 and VCAM-1 was also noted in a recent study on biopsy tissue of IC/PBS patients [91]. Increased expression of ICAM-1 subsequent to exposure with acetic acid in animal model is therefore clinically relevant.

Antisense experiments supported the earlier report that inhibition of NGF expression significantly downregulates the expression of ICAM-1 [80]. It is reported that binding of NGF to its high-affinity TrkA receptor controls the sICAM-1 expression on target cells [80]. Expression of ICAM-1 was
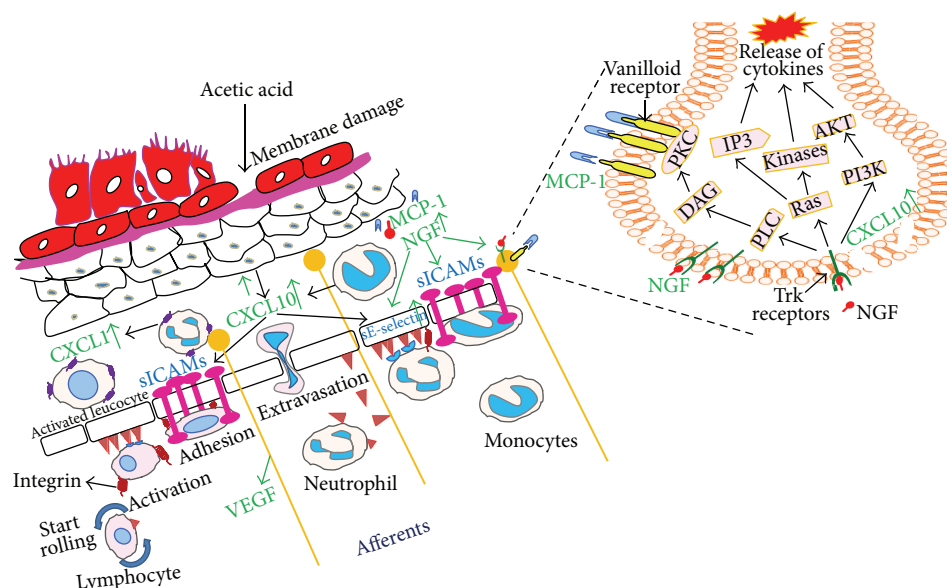
FIGURE 5: Signalling cascades induced by NGF overexpression following exposure to acetic acid. Injury from exposure to acetic sets off the signalling cascades that upregulate the expression of NGF, MCP-1, prostaglandins, CXCL-1, and CXCL-10. Overexpression of NGF drives the expression of VEGF from neurons and leptin from adipocytes covering neurons. NGF and MCP-1 also induce hyperexcitability in neurons by activating TRPV1 receptor. MCP-1, CXCL-1, and CXCL-10 cause chemoattraction of monocytes, neutrophils, and lymphocytes, which requires extravasation across endothelium from blood through the cooperation of adhesion molecules E-selectin and ICAM-1.

negatively associated with NGF expression in antisense experiments. Ultimately, expression of ICAM-1 is regulated at the level of transcription by one of several signaling cascades NF-κB, JAK/STAT, IFN-g (CXCL-10), AP, MAP kinase, and PKC [92].

Intravesical route can allow selective exposure of antisense ODN to the NGF producing cells in urothelium and avoid systemic side effects from genetic manipulation of NGF expression. Intravesical route can also prove to be cost effective given the cost of ODN or siRNA. Contrary to the goal of loss of function in bladder with antisense ODN, in recent studies liposomes have also been used to deliver ODN for gain of function in bladder [93].

10. Imaging of Intravesical Therapy

Drug development for DO and IC/PBS relies heavily on subjective outcomes for predicting efficacy and safety in early clinical development. Although there are many validated measurement tools used in DO and IC/PBS research, they are usually burdensome and do not capture all symptoms related to the lower urinary tract. Moreover, improvement in symptoms scores following an intervention does not always correlate with patient expectations, satisfaction, and goal achievement, which are critically important for successful management of DO and IC/PBS.

There is underdevelopment and underutilization of urine biomarkers and imaging methods for investigational drugs given by intravesical route. Overreliance on subjective impressions of patients for therapeutic response limits clinical relevance due to weak correlation with patient satisfaction and can also impede continued scientific progress in assessing

study outcomes relative to symptom bother. Symptom scores may also be related to the cause of high heterogeneity in clinical response in DO and IC/PBS patients.

Imaging can offer the possibility to provide region-specific information in situations where serum and urine assays may not reflect the true state of bladder [94–96]. Urine chemokines have been reported to be associated with symptom severity of IC/PBS patients [97]. Application of fluorescent microscopy for imaging of pelvic floor has been limited due to the issues pertaining to light absorption by tissues, scattering, and autofluorescence [98].

Recent studies have shown that dyes fluorescing in the near-infrared (NIR) band can overcome this handicap in deep-tissue imaging of experimental animals [99]. Selected NIR fluorochromes emit light with tissue penetration approaching 10–15 cm [100]. The light emission from probes in this spectrum encounters low background autofluorescence and minimal attenuation of signal due to light absorption by tissue components [98]. Therefore, NIR imaging yields high signal-to-noise ratios and is well suited for studying the distribution of instilled treatments in bladder (Figure 2(b)).

Preliminary studies suggest that imaging of mouse pelvic floor in NIR spectrum can be a viable option [101]. NIR imaging easily allows visualization and quantification of bladder distribution by tracing the migration of fluorescence-labeled liposomes in anaesthetized mouse. Compared with other biological assays, imaging could provide objective endpoint for patients with ulcerative cystitis to indicate its high translation potential. Other benefits of optical imaging include its relatively low cost compared with more traditional imaging systems such as MRI and PET. The instrument

consists of two near-infrared lasers emitting at 685 nm and 785 nm, camera, and novel optics.

Furthermore, preferential binding of liposomes and nanoparticles to bladder lesions of IC/PBS after instillation can be assessed by NIR imaging of bladder, where it can theoretically provide anatomically specific information about the real time status of ulcer/lesion in bladder surface or tissue abnormality [102]. Therefore, future studies can substitute a response variable that is continuous in nature instead of a subjective clinical outcome.

11. Conclusions

Adoption of forward-thinking approaches can achieve advancements in drug delivery systems targeted to improve pharmacotherapy of bladder diseases in the future. Latest developments in the field of nanotechnology can bring this mode of therapy from second line of treatment for refractory cases to the forefront of disease management. Liposomes are an attractive drug delivery platform by virtue of their biodegradability, biocompatibility, low toxicity, and simple and mild preparation methods.

Conflict of Interests

All authors except Dr. Michael Chancellor declare that there is no conflict of interests regarding the publication of this paper. Dr. Michael Chancellor owns stocks in the start-up liposome company, Lipella.

Acknowledgments

References

[1] J. Parkin, C. Shea, and G. R. Sant, "Intravesical dimethyl sulfoxide (DMSO) for interstitial cystitis—a practical approach," *Urology*, vol. 49, no. 5, pp. 105–107, 1997.

[2] C. Dowson, J. Watkins, M. S. Khan, P. Dasgupta, and A. Sahai, "Repeated botulinum toxin type A injections for refractory overactive bladder: medium-term outcomes, safety profile, and discontinuation rates," *European Urology*, vol. 61, no. 4, pp. 834–839, 2012.

[3] J. Kaufman, V. Tyagi, M. Anthony, M. B. Chancellor, and P. Tyagi, "State of the art in intravesical therapy for lower urinary tract symptoms," *Reviews in Urology*, vol. 12, pp. e181–e189, 2010.

[4] N. Yoshimura and M. B. Chancellor, "Current and future pharmacological treatment for overactive bladder," *Journal of Urology*, vol. 168, no. 5, pp. 1897–1913, 2002.

[5] K. A. Killinger, J. A. Boura, and K. M. Peters, "Pain in interstitial cystitis/bladder pain syndrome: do characteristics differ in ulcerative and non-ulcerative subtypes?" *International Urogynecology Journal*, vol. 24, no. 8, pp. 1295–1301, 2013.

[6] J. C. Nickel, P. Hanno, K. Kumar, and H. Thomas, "Second multicenter, randomized, double-blind, parallel-group evaluation of effectiveness and safety of intravesical sodium chondroitin

[7] P. M. Hanno, "Analysis of long-term elmiron therapy for interstitial cystitis," *Urology*, vol. 49, no. 5, pp. 93–99, 1997.

[8] A. Giannantoni, V. Bini, R. Dmochowski et al., "Reply to Claus Riedl's letter to the editor re: antonella Giannantoni, Vittorio Bini, Roger Dmochowski, et al. Contemporary management of the painful bladder: a systematic review," *European Urology*, vol. 61, no. 5, pp. 29–53, 2012.

[9] M. C. Lai, Y. C. Kuo, and H. C. Kuo, "Intravesical hyaluronic acid for interstitial cystitis/painful bladder syndrome: a comparative randomized assessment of different regimens," *International Journal of Urology*, vol. 20, pp. 203–207, 2013.

[10] J. Eldrup, J. Thorup, and S. L. Nielsen, "Permeability and ultrastructure of human bladder epithelium," *British Journal of Urology*, vol. 55, no. 5, pp. 488–492, 1983.

[11] C. L. Parsons, D. Boychuk, S. Jones, R. Hurst, and H. Callahan, "Bladder surface glycosaminoglycans: an epithelial permeability barrier," *Journal of Urology*, vol. 143, no. 1, pp. 139–142, 1990.

[12] Y. Kim, N. Yoshimura, H. Masuda, F. De Miguel, and M. B. Chancellor, "Antimuscarinic agents exhibit local inhibitory effects on muscarinic receptors in bladder-afferent pathways," *Urology*, vol. 65, no. 2, pp. 238–242, 2005.

[13] V. Tyagi, B. J. Philips, R. Su et al., "Differential expression of functional cannabinoid receptors in human bladder detrusor and urothelium," *Journal of Urology*, vol. 181, no. 4, pp. 1932–1938, 2009.

[14] B. J. Philips, M. C. Smaldone, V. L. Erickson, N. Yoshimura, M. B. Chancellor, and P. Tyagi, "Differential expression of neurokinin receptor subtypes in urothelium and detrusor of human bladder," *Journal of Urology*, vol. 179, article 354, 2008.

[15] S. A. Baker, G. W. Hennig, J. Han, F. C. Britton, T. K. Smith, and S. D. Koh, "Methionine and its derivatives increase bladder excitability by inhibiting stretch-dependent K+ channels," *British Journal of Pharmacology*, vol. 153, no. 6, pp. 1259–1271, 2008.

[16] J. M. Beckel, A. Kanai, S.-J. Lee, W. C. De Groat, and L. A. Birder, "Expression of functional nicotinic acetylcholine receptors in rat urinary bladder epithelial cells," *American Journal of Physiology—Renal Physiology*, vol. 290, no. 1, pp. F103–F110, 2006.

[17] L. A. Birder, A. J. Kanai, W. C. De Groat et al., "Vanilloid receptor expression suggests a sensory role for urinary bladder epithelial cells," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 23, pp. 13396–13401, 2001.

[18] P. Tyagi, C. A. Thomas, N. Yoshimura, and M. B. Chancellor, "Investigations into the presence of functional ß1, ß2 and ß3-adrenoceptors in urothelium and detrusor of human bladder," *International Brazilian Journal of Urology*, vol. 35, no. 1, pp. 76–83, 2009.

[19] A. T. Hanna-Mitchell, J. M. Beckel, S. Barbadora, A. J. Kanai, W. C. de Groat, and L. A. Birder, "Non-neuronal acetylcholine and urinary bladder urothelium," *Life Sciences*, vol. 80, no. 24-25, pp. 2298–2302, 2007.

[20] C. P. Smith, D. A. Gangitano, A. Munoz et al., "Botulinum toxin type A normalizes alterations in urothelial ATP and NO release induced by chronic spinal cord injury," *Neurochemistry International*, vol. 52, no. 6, pp. 1068–1075, 2008.

[21] Y.-C. Chuang, C. A. Thomas, S. Tyagi, N. Yoshimura, P. Tyagi, and M. B. Chancellor, "Human urine with solifenacin

intake but not tolterodine or darifenacin intake blocks detrusor overactivity," *International Urogynecology Journal and Pelvic Floor Dysfunction*, vol. 19, no. 10, pp. 1353–1357, 2008.

[22] Y. Kim, N. Yoshimura, H. Masuda, F. D. Miguel, and M. B. Chancellor, "Intravesical instillation of human urine after oral administration of trospium, tolterodine and oxybutynin in a rat model of detrusor overactivity," *BJU International*, vol. 97, no. 2, pp. 400–403, 2006.

[23] S. Tyagi, P. Tyagi, S. Van-le, N. Yoshimura, M. B. Chancellor, and F. de Miguel, "Qualitative and quantitative expression profile of muscarinic receptors in human urothelium and detrusor," *Journal of Urology*, vol. 176, no. 4, pp. 1673–1678, 2006.

[24] G. Gregoriadis and B. E. Ryman, "Liposomes as carriers of enzymes or drugs: a new approach to the treatment of storage diseases," *Biochemical Journal*, vol. 124, no. 5, p. 58, 1971.

[25] G. Gregoriadis, S. Jain, I. Papaioannou, and P. Laing, "Improving the therapeutic efficacy of peptides and proteins: a role for polysialic acids," *International Journal of Pharmaceutics*, vol. 300, no. 1-2, pp. 125–130, 2005.

[26] P. Sapra, P. Tyagi, and T. M. Allen, "Ligand-targeted liposomes for cancer treatment," *Current Drug Delivery*, vol. 2, no. 4, pp. 369–381, 2005.

[27] G. Gregoriadis, G. Dapergolas, and E. D. Neerunjun, "Penetration of target areas in the rat by liposome associated agents administered parenterally and intragastrically," *Biochemical Society Transactions*, vol. 4, no. 2, pp. 256–259, 1976.

[28] G. Gregoriadis, "Engineering liposomes for drug delivery: progress and problems," *Trends in Biotechnology*, vol. 13, no. 12, pp. 527–537, 1995.

[29] G. Gregoriadis and A. C. Allison, "Entrapment of proteins in liposomes prevents allergic reactions in pre immunised mice," *FEBS Letters*, vol. 45, no. 1, pp. 71–74, 1974.

[30] S. Lee, S. Dausch, C. Maierhofer, and D. Dausch, "A new therapy concept with a liposome eye spray for the treatment of the 'dry eye'," *Klinische Monatsblatter fur Augenheilkunde*, vol. 221, no. 10, pp. 825–836, 2004.

[31] D. Dausch, S. Lee, S. Dausch, J. C. Kim, G. Schwert, and W. Michelson, "Comparative study of treatment of the dry eye syndrome due to disturbances of the tear film lipid layer with lipid-containing tear substitutes: efficacy of lipid-containing tear substitutes," *Klinische Monatsblatter fur Augenheilkunde*, vol. 223, no. 12, pp. 974–983, 2006.

[32] S. Ebrahim, G. A. Peyman, and P. J. Lee, "Applications of liposomes in ophthalmology," *Survey of Ophthalmology*, vol. 50, no. 2, pp. 167–182, 2005.

[33] J. Nirmal, P. Tyagi, L. Dang et al., "Endocytosis uptake of liposomes in urothelium cells detected by transmission electron microscopy," *The Journal of Urology*, vol. 187, article e15, 2012.

[34] D. N. Frangos, J. J. Killion, D. Fan, R. Fishbeck, A. C. Von Eschenbach, and I. J. Fidler, "The development of liposomes containing interferon alpha for the intravesical therapy of human superficial bladder cancer," *Journal of Urology*, vol. 143, no. 6, pp. 1252–1266, 1990.

[35] J. W. Johnson, R. Nayar, J. J. Killion, A. C. Von Eschenbach, and I. J. Fidler, "Binding of liposomes to human bladder tumor epithelial cell lines: implications for an intravesical drug delivery system for the treatment of bladder cancer," *Selective Cancer Therapeutics*, vol. 5, no. 4, pp. 147–155, 1989.

[36] Y.-C. Chuang, M. B. Chancellor, S. Seki et al., "Intravesical protamine sulfate and potassium chloride as a model for bladder hyperactivity," *Urology*, vol. 61, no. 3, pp. 664–670, 2003.

[37] J. Kaufman, P. Tyagi, and M. B. Chancellor, "Intravesical liposomal (Lp08) instillation protects bladder urothelium from chemical irritation," *The Journal of Urology*, vol. 181, article 539, 2009.

[38] P. Tyagi, M. B. Chancellor, Z. Li et al., "Urodynamic and immunohistochemical evaluation of intravesical capsaicin delivery using thermosensitive hydrogel and liposomes," *Journal of Urology*, vol. 171, no. 1, pp. 483–489, 2004.

[39] M. O. Fraser, Y.-C. Chuang, P. Tyagi et al., "Intravesical liposome administration—a novel treatment for hyperactive bladder in the rat," *Urology*, vol. 61, no. 3, pp. 656–663, 2003.

[40] P. Tyagi, M. Chancellor, N. Yoshimura, and L. Huang, "Activity of different phospholipids in attenuating hyperactivity in bladder irritation," *BJU International*, vol. 101, no. 5, pp. 627–632, 2008.

[41] Y. Sun and T. C. Chai, "Effects of dimethyl sulphoxide and heparin on stretch-activated ATP release by bladder urothelial cells from patients with interstitial cystitis," *BJU International*, vol. 90, no. 4, pp. 381–385, 2002.

[42] J. J. Bade, "A placebo-controlled study of intravesical pentosanpolysulphate for the treatment of interstitial cystitis," *British Journal of Urology*, vol. 79, no. 2, pp. 168–171, 1997.

[43] P. Tyagi, V. C. Hsieh, N. Yoshimura, J. Kaufman, and M. B. Chancellor, "Instillation of liposomes vs dimethyl sulphoxide or pentosan polysulphate for reducing bladder hyperactivity," *BJU International*, vol. 104, no. 11, pp. 1689–1692, 2009.

[44] W.-C. Lee, C.-T. Chien, H.-J. Yu, and S.-W. Lee, "Bladder dysfunction in rats with metabolic syndrome induced by long-term fructose feeding," *Journal of Urology*, vol. 179, no. 6, pp. 2470–2476, 2008.

[45] J. I. Gillespie, G. A. Van Koeveringe, S. G. De Wachter, and J. De Vente, "On the origins of the sensory output from the bladder: the concept of afferent noise," *BJU International*, vol. 103, no. 10, pp. 1324–1333, 2009.

[46] W. C. Lee, P. H. Chiang, Y. L. Tain, C. C. Wu, and Y. C. Chuang, "Sensory dysfunction of bladder mucosa and bladder oversensitivity in a rat model of metabolic syndrome," *PloS ONE*, vol. 7, Article ID e45578, 2012.

[47] Y.-C. Chuang, W.-C. Lee, W.-C. Lee, and P.-H. Chiang, "Intravesical liposome versus oral pentosan polysulfate for interstitial cystitis/painful bladder syndrome," *Journal of Urology*, vol. 182, no. 4, pp. 1393–1400, 2009.

[48] W.-C. Lee, Y.-C. Chuang, W.-C. Lee, and P.-H. Chiang, "Safety and dose flexibility clinical evaluation of intravesical liposome in patients with interstitial cystitis or painful bladder syndrome," *Kaohsiung Journal of Medical Sciences*, vol. 27, no. 10, pp. 437–440, 2011.

[49] S. T. Truschel, E. Wang, W. G. Ruiz et al., "Stretch-regulated exocytosis/endocytosis in bladder umbrella cells," *Molecular Biology of the Cell*, vol. 13, no. 3, pp. 830–846, 2002.

[50] M. B. Chancellor and W. C. De Groat, "Intravesical capsaicin and resiniferatoxin therapy: spicing up the ways to treat the overactive bladder," *Journal of Urology*, vol. 162, no. 1, pp. 3–11, 1999.

[51] D. S. Byrne, A. Das, J. Sedor et al., "Effect of intravesical capsaicin and vehicle on bladder integrity in control and spinal cord injured rats," *Journal of Urology*, vol. 159, no. 3, pp. 1074–1078, 1998.

[52] M. Mandal and K.-D. Lee, "Listeriolysin O-liposome-mediated cytosolic delivery of macromolecule antigen in vivo: enhancement of antigen-specific cytotoxic T lymphocyte frequency,

activity, and tumor protection," *Biochimica et Biophysica Acta*, vol. 1563, no. 1-2, pp. 7–17, 2002.

[53] Y.-C. Chuang, P. Tyagi, C.-C. Huang et al., "Urodynamic and immunohistochemical evaluation of intravesical botulinum toxin a delivery using liposomes," *Journal of Urology*, vol. 182, no. 2, pp. 786–792, 2009.

[54] Y.-C. Chuang, N. Yoshimura, C.-C. Huang, P.-H. Chiang, and M. B. Chancellor, "Intravesical botulinum toxin a administration produces analgesia against acetic acid induced bladder pain responses in rats," *Journal of Urology*, vol. 172, no. 4, pp. 1529–1532, 2004.

[55] S. P. Petrou, A. S. Parker, J. E. Crook, A. Rogers, D. Metz-Kudashick, and D. D. Thiel, "Botulinum A toxin/dimethyl sulfoxide bladder instillations for women with refractory idiopathic detrusor overactivity: a phase 1/2 study," *Mayo Clinic Proceedings*, vol. 84, no. 8, pp. 702–706, 2009.

[56] P. Caccin, O. Rossetto, M. Rigoni, E. Johnson, G. Schiavo, and C. Montecucco, "VAMP/synaptobrevin cleavage by tetanus and botulinum neurotoxins is strongly enhanced by acidic liposomes," *FEBS Letters*, vol. 542, no. 1–3, pp. 132–136, 2003.

[57] M. L. Stephenson and P. C. Zamecnik, "Inhibition of Rous sarcoma viral RNA translation by a specific oligodeoxyribonucleotide," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 75, no. 1, pp. 285–288, 1978.

[58] H. Dadgostar and N. Waheed, "The evolving role of vascular endothelial growth factor inhibitors in the treatment of neovascular age-related macular degeneration," *Eye*, vol. 22, no. 6, pp. 761–767, 2008.

[59] C. Gebhard, G. Huard, E. A. Kritikou, and J. C. Tardif, "Apolipoprotein B antisense inhibition—update on mipomersen," *Current Pharmaceutical Design*, vol. 919, no. 17, pp. 3132–3142, 2013.

[60] T. Yokota, E. Hoffman, and S. Takeda, "Antisense oligo-mediated multiple exon skipping in a dog model of duchenne muscular dystrophy," *Methods in Molecular Biology*, vol. 709, pp. 299–312, 2011.

[61] B. L. Jacobs, M. C. Smaldone, V. Tyagi et al., "Increased nerve growth factor in neurogenic overactive bladder and interstitial cystitis patients," *The Canadian Journal of Urology*, vol. 17, no. 1, pp. 4989–4994, 2010.

[62] H.-T. Liu and H.-C. Kuo, "Intravesical botulinum toxin A injections plus hydrodistension can reduce nerve growth factor production and control bladder pain in interstitial cystitis," *Urology*, vol. 70, no. 3, pp. 463–468, 2007.

[63] H. T. Liu and H. C. Kuo, "Increased urine and serum nerve growth factor levels in interstitial cystitis suggest chronic inflammation is involved in the pathogenesis of disease," *PloS ONE*, vol. 7, Article ID e44687, 2012.

[64] N. Yoshimura, N. E. Bennett, Y. Hayashi et al., "Bladder overactivity and hyperexcitability of bladder afferent neurons after intrathecal delivery of nerve growth factor in rats," *Journal of Neuroscience*, vol. 26, no. 42, pp. 10847–10855, 2006.

[65] S. Seki, K. Sasaki, Y. Igawa et al., "Suppression of detrusor-sphincter dyssynergia by immunoneutralization of nerve growth factor in lumbosacral spinal cord in spinal cord injured rats," *Journal of Urology*, vol. 171, no. 1, pp. 478–482, 2004.

[66] R. J. Evans, R. M. Moldwin, N. Cossons, A. Darekar, I. W. Mills, and D. Scholfield, "Proof of concept trial of tanezumab for the treatment of symptoms associated with interstitial cystitis," *Journal of Urology*, vol. 185, no. 5, pp. 1716–1721, 2011.

[67] P. Tyagi, R. Banerjee, S. Basu, N. Yoshimura, M. Chancellor, and L. Huang, "Intravesical antisense therapy for cystitis using TAT-peptide nucleic acid conjugates," *Molecular Pharmaceutics*, vol. 3, no. 4, pp. 398–406, 2006.

[68] D. C. Molliver, D. E. Wright, M. L. Leitner et al., "IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life," *Neuron*, vol. 19, no. 4, pp. 849–861, 1997.

[69] M. Tyagi, M. Rusnati, M. Presta, and M. Giacca, "Internalization of HIV-1 tat requires cell surface heparan sulfate proteoglycans," *Journal of Biological Chemistry*, vol. 276, no. 5, pp. 3254–3261, 2001.

[70] E. Uhlmann, A. Ryte, and A. Peyman, "Studies on the mechanism of stabilization of partially phosphorothioated oligonucleotides against nucleolytic degradation," *Antisense and Nucleic Acid Drug Development*, vol. 7, no. 4, pp. 345–350, 1997.

[71] M. Nogawa, T. Yuasa, S. Kimura et al., "Intravesical administration of small interfering RNA targeting PLK-1 successfully prevents the growth of bladder cancer," *Journal of Clinical Investigation*, vol. 115, no. 4, pp. 978–985, 2005.

[72] M. Kashyap, N. Kawamorita, V. Tyagi et al., "Downregulation of NGF expression in the bladder by antisense oligoucleotides as new treatment for overactive bladder," *Journal of Urology*, vol. 27190, no. 2, pp. 757–764, 2013.

[73] C.-J. Arum, Y. Kodama, N. Rolim et al., "A rat model of intravesical delivery of small interfering RNA for studying urinary carcinoma," *World Journal of Urology*, vol. 28, no. 4, pp. 479–485, 2010.

[74] C. E. Blietz, B. Thode, M. Hauses et al., "In vivo studies on the availability and toxicity of antisense oligonucleotides in bladder cancer," *In Vivo*, vol. 23, no. 1, pp. 13–19, 2009.

[75] D. Oddiah, P. Anand, S. B. McMahon, and M. Rattray, "Rapid increase of NGF, BDNF and NT-3 mRNAs in inflamed bladder," *NeuroReport*, vol. 9, no. 7, pp. 1455–1458, 1998.

[76] M. R. Saban, H. Hellmich, N. B. Nguyen, J. Winston, T. G. Hammond, and R. Saban, "Time course of LPS-induced gene expression in a mouse model of genitourinary inflammation," *Physiol Genomics*, vol. 5, no. 3, pp. 147–160, 2001.

[77] B. Schnegelsberg, T. T. Sun, G. Cain et al., "Overexpression of NGF in mouse urothelium leads to neuronal hyperinnervation, pelvic sensitivity, and changes in urinary bladder function," *The American Journal of Physiology—Regulatory, Integrative and Comparative Physiology*, vol. 298, no. 5, pp. R534–R547, 2010.

[78] S. Cardenas, M. Scuri, L. Samsell et al., "Neurotrophic and neuroimmune responses to early-life Pseudomonas aeruginosa infection in rat lungs," *American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 299, no. 3, pp. L334–L344, 2010.

[79] K. Nakamura, F. Tan, Z. Li, and C. J. Thiele, "NGF activation of TrkA induces vascular endothelial growth factor expression via induction of hypoxia-inducible factor-1α," *Molecular and Cellular Neuroscience*, vol. 46, no. 2, pp. 498–506, 2011.

[80] S. Othumpangat, M. Regier, and G. Piedimonte, "Nerve growth factor modulates human rhinovirus infection in airway epithelial cells by controlling ICAM-1 expression," *American Journal of Physiology Lung Cellular and Molecular Physiology*, vol. 15302, pp. L1057–L1066, 2012.

[81] H. Jung, P. T. Toth, F. A. White, and R. J. Miller, "Monocyte chemoattractant protein-1 functions as a neuromodulator in dorsal root ganglia neurons," *Journal of Neurochemistry*, vol. 104, no. 1, pp. 254–263, 2008.

[82] F. A. White, H. Jung, and R. J. Miller, "Chemokines and the pathophysiology of neuropathic pain," *Proceedings of the*

National Academy of Sciences of the United States of America, vol. 104, no. 51, pp. 20151–20158, 2007.

[83] G. Banisadr, R.-D. Gosselin, P. Mechighel, W. Rostène, P. Kitabgi, and S. M. Parsadaniantz, "Constitutive neuronal expression of CCR2 chemokine receptor and its colocalization with neurotransmitters in normal rat brain: functional effect of MCP-1/CCL2 on calcium mobilization in primary cultured neurons," *Journal of Comparative Neurology*, vol. 492, no. 2, pp. 178–192, 2005.

[84] S. Bhangoo, D. Ren, R. J. Miller et al., "Delayed functional expression of neuronal chemokine receptors following focal nerve demyelination in the rat: a mechanism for the development of chronic sensitization of peripheral nociceptors," *Molecular Pain*, vol. 3, article 38, 2007.

[85] T. Maeda, N. Kiguchi, Y. Kobayashi, T. Ikuta, M. Ozaki, and S. Kishioka, "Leptin derived from adipocytes in injured peripheral nerves facilitates development of neuropathic pain via macrophage stimulation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 31, pp. 13076–13081, 2009.

[86] H. Kajiwara, M. Yasuda, N. Kumaki, T. Shibayama, and Y. Osamura, "Expression of carbohydrate antigens (SSEA-1, Sialyl-Lewis X, DU-PAN-2 and CA19-9) and E-selectin in urothelial carcinoma of the renal pelvis, ureter, and urinary bladder," *Tokai Journal of Experimental and Clinical Medicine*, vol. 30, no. 3, pp. 177–182, 2005.

[87] A. M. S. Assreuy, G. J. Martins, M. E. F. Moreira et al., "Prevention of cyclophosphamide-induced hemorrhagic cystitis by glucose- mannose binding plant lectins," *Journal of Urology*, vol. 161, no. 6, pp. 1988–1993, 1999.

[88] S. M. Vinson, A. Rickard, J. S. Ryerse, and J. McHowat, "Neutrophil adherence to bladder microvascular endothelial cells following platelet-activating factor acetylhydrolase inhibition," *Journal of Pharmacology and Experimental Therapeutics*, vol. 314, no. 3, pp. 1241–1247, 2005.

[89] M. Green, A. Filippou, G. Sant, and T. C. Theoharides, "Expression of intercellular adhesion molecules in the bladder of patients with interstitial cystitis," *Urology*, vol. 63, no. 4, pp. 688–693, 2004.

[90] M. Leppilahti, P. Hellström, and T. L. J. Tammela, "Effect of diagnostic hydrodistension and four intravesical hyaluronic acid instillations on bladder ICAM-1 intensity and association of ICAM-1 intensity with clinical response in patients with interstitial cystitis," *Urology*, vol. 60, no. 1, pp. 46–51, 2002.

[91] A. T. Corcoran, N. Yoshimura, V. Tyagi, B. Jacobs, W. Leng, and P. Tyagi, "Mapping the cytokine profile of painful bladder syndrome/interstitial cystitis in human bladder and urine specimens," *World Journal of Urology*, vol. 31, no. 1, pp. 241–246, 2013.

[92] S. Garrean, X.-P. Gao, V. Brovkovych et al., "Caveolin-1 regulates NF-κB activation and lung inflammatory response to sepsis induced by lipopolysaccharide," *Journal of Immunology*, vol. 177, no. 7, pp. 4853–4860, 2006.

[93] M. R. Kang, G. Yang, R. F. Place et al., "Intravesical delivery of small activating RNA formulated into lipid nanoparticles inhibits orthotopic bladder tumor growth," *Cancer Research*, vol. 172, pp. 5069–5079, 2012.

[94] P. Tyagi, D. Barclay, R. Zamora et al., "Urine cytokines suggest an inflammatory response in the overactive bladder: a pilot study," *International Urology and Nephrology*, vol. 42, no. 3, pp. 629–635, 2010.

[95] P. Tyagi, B. L. Jacobs, D. Barcaly et al., "Urine levels of inflammatory chemokines can be novel biomarkers for the overactive bladder," *The Journal of Urology*, vol. 181, article 588, 2009.

[96] P. Tyagi, V. Tyagi, D. Bui et al., "Discrimination Of OAB from IC/PBS by multivariate data modeling of urinary proteins," *The Journal of Urology*, vol. 183, pp. e614–e6e5, 2010.

[97] P. Tyagi, D. Nikolavsky, Y. Vodovotz et al., "Urine levels of selected chemokines positively correlate with lower bladder capacity and psychometric scores in Ic/Pbs patients," *The Journal of Urology*, vol. 181, article 21, 2009.

[98] R. Weissleder and V. Ntziachristos, "Shedding light onto live molecular targets," *Nature Medicine*, vol. 9, no. 1, pp. 123–128, 2003.

[99] C. Bremer, V. Ntziachristos, and R. Weissleder, "Optical-based molecular imaging: contrast agents and potential medical applications," *European Radiology*, vol. 13, no. 2, pp. 231–243, 2003.

[100] U. Mahmood and R. Weissleder, "Near-infrared optical imaging of proteases in cancer," *Molecular Cancer Therapeutics*, vol. 2, pp. 489–496, 2003.

[101] J. Kaufman, H. Hensley, J. Jacobs et al., "Non-invasive imaging of near infrafred dye labeled liposomes facilitates evaluation of bioresidence time," *The Journal of Urology*, vol. 183, article e628, 2010.

[102] Y.-X. J. Wang, "Medical imaging in pharmaceutical clinical trials: what radiologists should know," *Clinical Radiology*, vol. 60, no. 10, pp. 1051–1057, 2005.
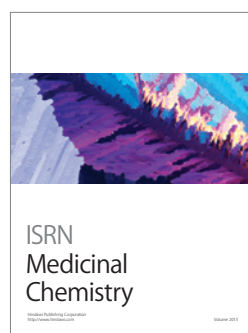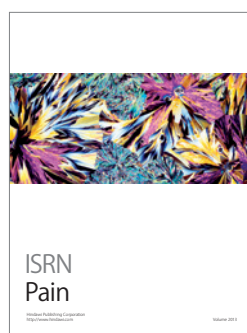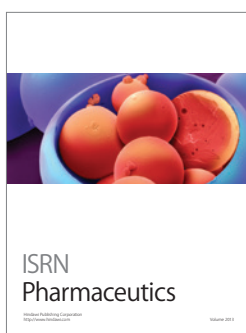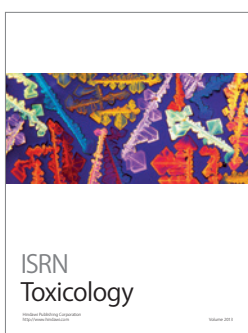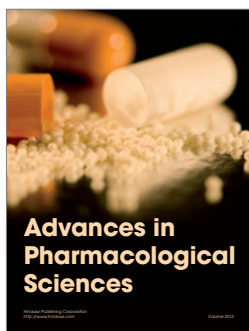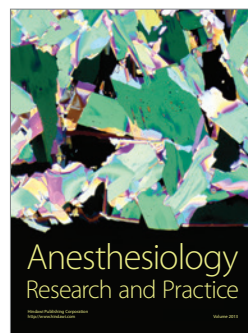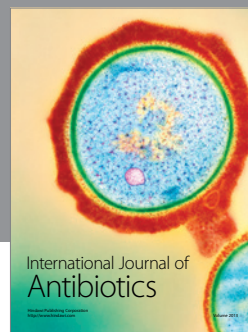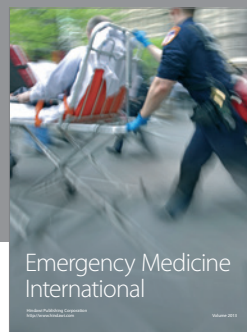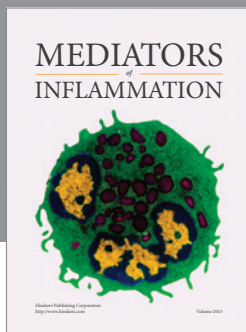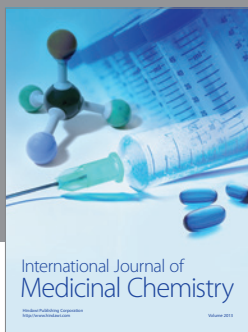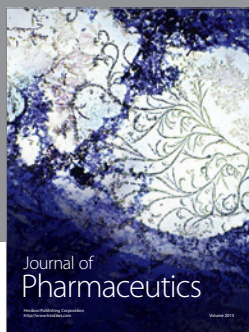
ORIGINAL ARTICLE

Cross-sensitization mechanisms between colon and bladder via transient receptor potential A1 stimulation in rats

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Abstract

Introduction and hypothesis The aim of this study was to analyze the mechanism underlying cross-sensitization between the colon and the bladder via activation of transient receptor potential A1 (TRPA1) channels.
Methods Using female Sprague–Dawley rats, polyethylene catheters were inserted into the colon between two ligations at the levels of 40 and 60 mm rostral to the anus and into the bladder. (1) We examined changes in colon and bladder activity after the application of allyl isothiocyanate (AI, 50 mM, 300 µl), a TRPA1 activator, into the colon or the bladder in an awake condition. Inhibitory effects of the pretreatment with HC-030031 (HC, 3 mg/kg), a TRPA1 inhibitor, on colon-to-bladder cross-sensitization induced by AI instilled in the colon were also investigated. (2) We examined Evans blue (EB) dye extravasation after TRPA1 stimulation in the colon or the bladder to evaluate vascular permeability due to tissue inflammation.
Results (1) Intercontraction intervals during continuous saline infusion into the bladder (0.04 ml/min) were significantly decreased after the intracolonic AI application, which significantly increased mean intracolonic pressure, indicative of colon-to-bladder cross-sensitization. The AI-induced colon-to-bladder cross-sensitization was completely prevented by the

pretreatment with intravenous application of HC. On the other hand, mean intracolonic pressure was significantly decreased after the intravesical AI application, which significantly increased mean intravesical pressure. (2) EB dye extravasation was significantly increased in the AI-treated inflamed organs and also in the bladder following intracolonic AI treatment.
Conclusions Colon-to-bladder cross-sensitization is mediated via TRPA1 stimulation in the colon, although TRPA1 expressed in the bladder does not seem to participate in bladder-to-colon cross-sensitization.

Introduction

Chronic pelvic pain is a major symptom of many complex clinical conditions, including interstitial cystitis/bladder pain syndrome (IC/BPS) and irritable bowel syndrome (IBS). In fact, over one third of patients diagnosed with IC/BPS also exhibit symptoms of IBS [1, 2], and conversely 26–56 % of patients diagnosed with IBS also have symptoms of IC/BPS [3]. However, while the pathogenesis of IC/BPS, IBS, and other chronic pelvic pain disorders has mostly been studied independently, few investigators have examined multiorgan mechanisms in the development of chronic pelvic pain despite their striking clinical overlap. The high concurrence rate of chronic pain disorders supports a role of sensitized convergent sensory pathways in peripheral and central nervous systems, which may occur through infectious, inflammatory, neurogenic, metabolic, or other neuropathic mechanisms [4].

Among various noxious stimulus detectors, the transient receptor potential (TRP) channel family is the largest group, which consists of a large number of cation channels and is divided into six subfamilies: TRPV (vanilloid), TRPA

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(ankyrin), TRPM (melastatin), TRPC (canonical), TRPP (polycystin), and TRPML (Mucolipin) in mammals. In the TRP family, TRPA1, TRPV1, TRPV4, and TRPM8 channels are expressed in nociceptive sensory neurons and contribute to the detection of noxious mechanical, thermal, and/or chemical stimuli [5–7]. TRPV1 is the best characterized channel of the TRP family in terms of expression pattern, properties, and clinical translation of its manipulation [8]. On the other hand, TRPA1 is a more recently identified member of the TRP family that has emerged as a major mediator of inflammatory pain and is selectively expressed by a subset of nociceptive sensory neurons that also express TRPV1 [5], suggesting that TRPV1 and TRPA1 may mutually control the transduction of noxious stimuli in TRPV1-expressing sensory neurons [9]. On the other hand, there are no apparent overlaps in the localization of other TRP channels such as TRPV4, TRPM8, and TRPV1 [5, 6, 10].

Increased TRPV1 expression in the bladder or the colon has been found in patients with chronic pelvic pain [11, 12]. However, the pathogenesis of chronic pelvic pain remains unknown, and there are few animal studies to analyze TRP channels involved in IC/BPS and/or IBS via pelvic organ cross-sensitization. It has also been reported in rats that intracolonic application of trinitrobenzene sulfonic acid (TNBS) induces a reduction of bladder capacity, which is abolished by intracolonic pretreatment with resiniferatoxin that induces desensitization of TRPV1-expressing afferents in the colon [13], suggesting that TRPV1 in the colon may be involved in colon-to-bladder cross-sensitization via convergence of TRPV1-expressing pelvic afferents. In addition, mustard oil (allyl isothiocyanate, AI), TNBS, and acrolein (the metabolite of cyclophosphamide) have often been used to examine the neural mechanisms of pelvic organ cross-sensitization [14], and these irritant chemicals are known to be the activator of TRPA1 channels [15–17], raising a possibility that TRPA1 in TRPV1-expressing sensory neurons may contribute to the pathogenesis of chronic pelvic pain via pelvic organ cross-sensitization. Thus, we analyzed the mechanism of cross-sensitization between the colon and the bladder following TRPA1 stimulation in the colon or the bladder.

Materials and methods

Forty female Sprague–Dawley rats weighing 200–250 g were used in this study. Care and handling of animals were in accordance with institutional guidelines, and the protocol was approved by the Animal Ethics Committee of Jikei University School of Medicine. Under isoflurane anesthesia, the colon and the bladder were exposed through a lower midline abdominal incision. Feces were gently removed from the distal colon through a small incision at the level of 70 mm rostral to the anus, and the incision was closed. The distal colon was then ligated with 5-0 silk sutures at the levels of 40

and 60 mm rostral to the anus, and a polyethylene-50 (PE-50) catheter was implanted between two sutures. A PE-50 catheter was also implanted into the bladder from the bladder dome. The implanted catheters were exteriorized through the abdominal wall, and the wound was closed with sutures.

After the surgery, the animals were placed in a sling-suit harness (Lomir Biomedical, Malone, NY, USA) and allowed to recover from the anesthesia for 1 h, so that cystometry was performed in an awake condition. To analyze colon-to-bladder cross-sensitization, the intravesical catheter was connected via a three-way stopcock to a pressure transducer (ADInstruments, Castle Hill, NSW, Australia) and a syringe pump (KD Scientific, Holliston, MA, USA). Saline solution was infused at 0.04 ml/min for approximately 1 h until rhythmic bladder contractions became stable. Cystometric parameters were first measured for 2 h after vehicle (saline, 300 μl) application into the colon and, thereafter, for another 2 h after intracolonic application of AI (50 mM, 0.5 %, 300 μl), a TRPA1 activator (n=8). Baseline pressure, voiding threshold pressure, maximal voiding pressure, intercontraction interval, and mean intracolonic pressure were recorded using data acquisition software (sampling at 10 Hz, Chart, ADInstruments) on a computer system equipped with an analog-to-digital converter (PowerLab, ADInstruments). In a separate group of animals, a PE-10 catheter was implanted in the right jugular vein under isoflurane anesthesia. To determine whether the AI-induced colon-to-bladder cross-sensitization is induced by TRPA1 stimulation, HC-030031 (HC, 3 mg/kg, 0.5 ml/kg), a TRPA1 inhibitor, was intravenously (i.v.) applied 30 min before intracolonic AI administration (n=8). The concentration of AI and HC was selected based on previous studies [18, 19] and our preliminary experiments.

Next, in another group of rats, PE-50 catheters were inserted between two ligations of the colon and into the bladder under isoflurane anesthesia as described above. The bladder neck was also ligated with 5-0 silk sutures to prevent leakage of AI injected into the bladder. Saline solution (300 μl) was injected into the colon ligated both sites to monitor the intracolonic pressure. To analyze the bladder-to-colon cross-sensitization, mean intracolonic pressure and mean intravesical pressure were measured for each 2-h period after vehicle (saline, 300 μl) application and the subsequent application of AI (50 mM, 300 μl) into the bladder (n=8).

Finally, in a separate group of animals, two sites of the colon, and the bladder neck, were ligated with 5-0 silk sutures as described above under urethane (1.2 g/kg, subcutaneously) anesthesia. AI (50 mM, 300 μl) or vehicle (saline, 300 μl) was injected into the colon to determine the colon-to-bladder cross-sensitization or into the bladder to examine the bladder-to-colon cross-sensitization via a 23-gauge needle inserted into the respective organ (each n=8). The needle was then removed and the injection site was immediately compressed by a cotton swab for 1 min. After no leakage of

the solution was confirmed, the abdominal wound was closed. Two hours after the AI application into the colon or the bladder, vascular permeability due to tissue inflammation was evaluated by injection of Evans blue (EB, 50 mg/kg, 0.5 ml/kg) dye into the jugular vein [20]. The colon and the bladder were dissected 30 min after the EB dye injection and dried at 50 °C for 24 h. Dried tissues were weighed and immersed in tubes containing 1 ml formamide for 72 h. The amount of dye extracted from the tissues was then determined in duplicate using a microplate reader (Model 680, Bio-Rad Laboratories, Hercules, CA, USA) set at 620 nm wavelength, and the results are expressed as micrograms of EB per dry weight of each tissue specimen. AI, HC, and EB were purchased from Sigma (St. Louis, MO, USA). AI and EB were dissolved in saline solution, and HC was dissolved in 10 % dimethyl sulfoxide, 10 % Tween 80, and 80 % saline solution.

All data were represented as mean values ± standard error of the mean. Statistical analysis software (Prism, GraphPad Software, San Diego, CA, USA) was used to compare the results. Time-dependent changes in cystometric parameters (baseline pressure, voiding threshold pressure, maximal voiding pressure, intercontraction interval), mean intracolonic pressure, and mean intravesical pressure were analyzed using one-way repeated measures analysis of variance (ANOVA) followed by Tukey's multiple comparison test. The comparison of EB concentration between AI and vehicle groups was

analyzed using unpaired Student's t test. A p value <0.05 was regarded as statistically significant.

Results

In the colon-to-bladder cross-sensitization experiments, there were no apparent changes in any cystometric parameters or colonic pressure for 2 h after vehicle (saline) injection into the colon (Fig. 1a). However, following the application of AI into the colon, mean intracolonic pressure was significantly increased from 30 min after the application (0 min: 5.1 ± 2.0 cmH$_2$O, 30 min: 14.4 ± 6.0 cmH$_2$O, 60 min: 22.2 ± 8.8 cmH$_2$O, 90 min: 26.1 ± 13.0 cmH$_2$O, and 120 min: 29.1 ± 17.3 cmH$_2$O) (Figs. 1a and 2a). Also, in bladder activity measurement, voiding threshold pressure and intercontraction interval were gradually decreased (voiding threshold pressure, 0 min: 8.0 ± 1.3 cmH$_2$O, 30 min: 7.6 ± 1.2 cmH$_2$O, 60 min: 7.3 ± 1.0 cmH$_2$O, 90 min: 6.9 ± 1.0 cmH$_2$O, and 120 min: 6.9 ± 1.0 cmH$_2$O; intercontraction interval, 0 min: 244.6 ± 68.6 s, 30 min: 240.6 ± 70.4 s, 60 min: 216.0 ± 49.8 s, 90 min: 178.8 ± 42.9 s, and 120 min: 156.4 ± 38.4 s) with a significant decrease from 90 min (Figs. 1a and 2a), whereas there were no significant time-dependent changes in baseline pressure and maximal voiding pressure, indicative of colon-to-bladder cross-sensitization. However, when HC was applied i.v. 30 min

Fig. 1 Representative traces showing changes in intravesical and intracolonic pressures after allyl isothiocyanate (AI), a TRPA1 activator, was intracolonically (i.col.) (a) or intravesically (i.ves.) (b) applied. a Bladder overactivity shown by frequent bladder contractions during continuous saline infusion (0.04 ml/min) into the bladder was induced by TRPA1 stimulation in the colon (AI placement), indicative of colon-to-bladder cross-sensitization. There was no apparent change in bladder activity when saline solution was injected and retained in the colon (vehicle placement) prior to AI administration. b Bladder-to-colon cross-sensitization was not seen after TRPA1 stimulation in the bladder (AI placement). Colon activity was also not affected by saline injection into the bladder (vehicle placement) prior to AI administration
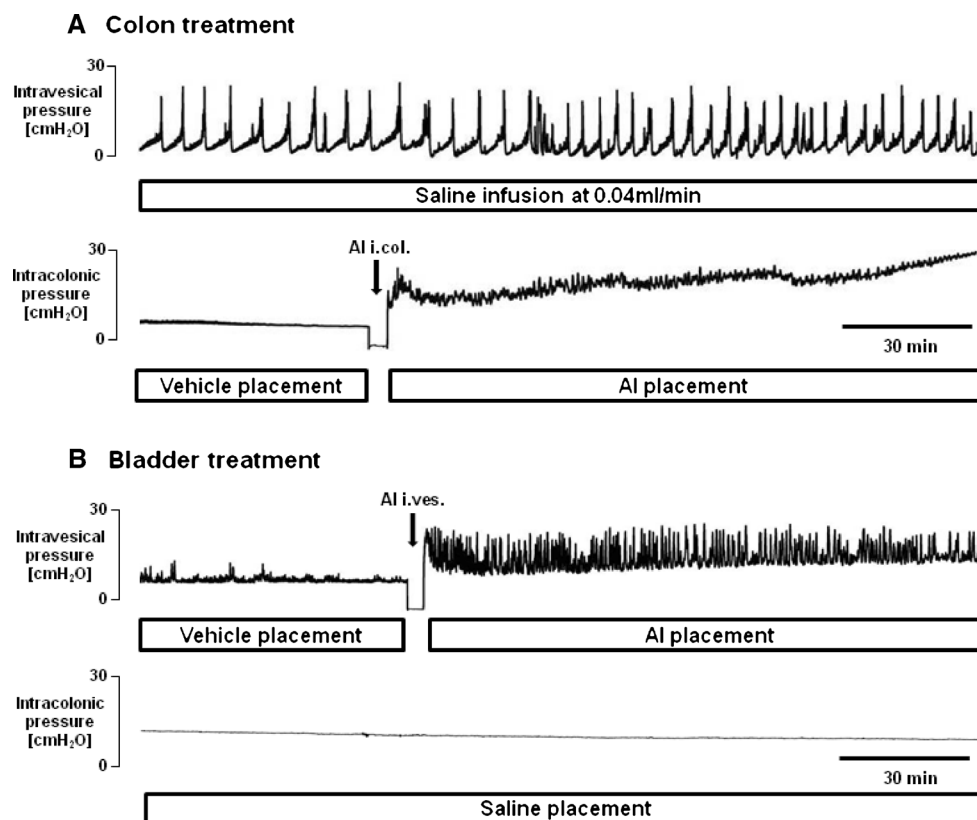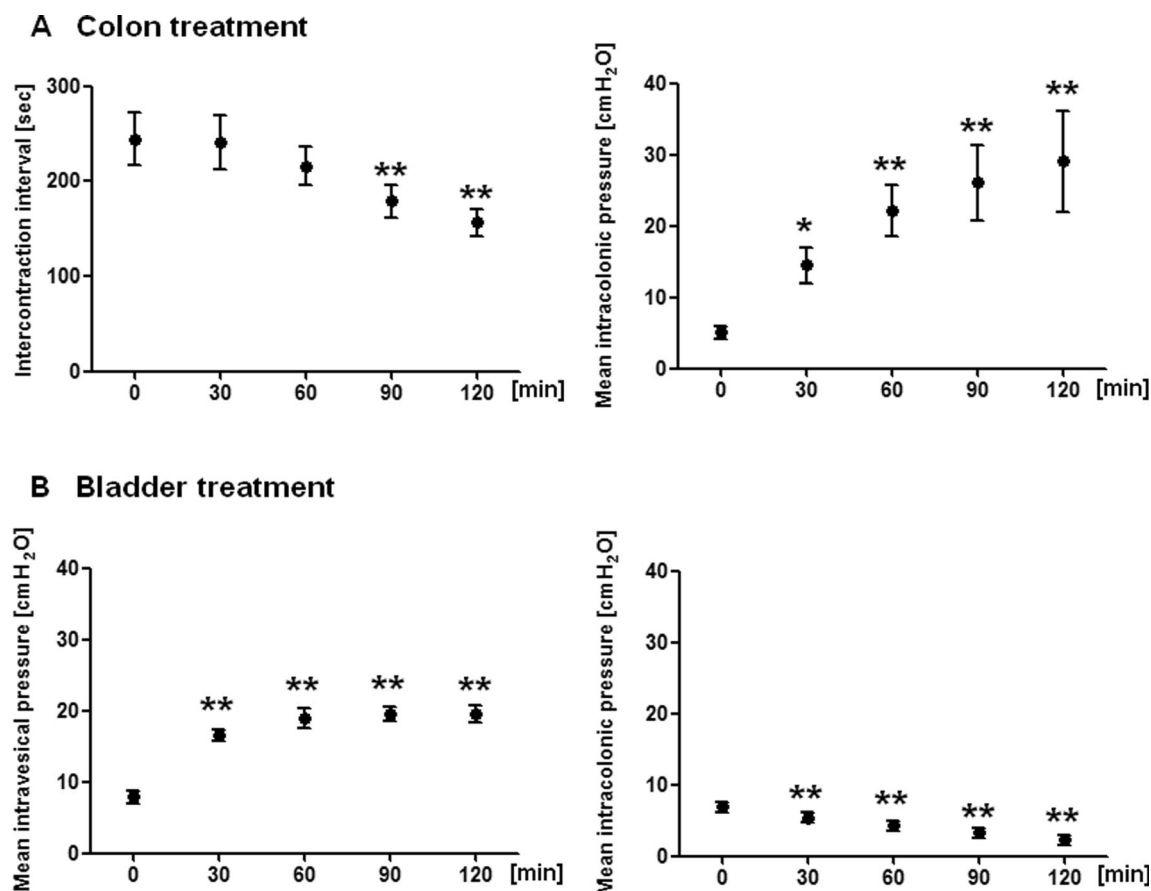
Fig. 2 Time-dependent changes in intercontraction interval, mean intracolonic pressure, and mean intravesical pressure after application of AI, a TRPA1 activator, into the colon (**a**) or the bladder (**b**). **a** After the application of AI into the colon, mean intracolonic pressure was significantly increased from 30 min and intercontraction interval was significantly decreased from 90 min, indicative of colon-to-bladder cross-sen-

sitization. **b** After the application of AI into the bladder, mean intracolonic pressure was significantly decreased from 30 min, although mean intravesical pressure was significantly increased from 30 min, indicating that bladder-to-colon cross-sensitization was not induced by TRPA1 stimulation in the bladder (**b**). $*p<0.05$ and $**p<0.01$

before intracolonic AI administration, significant changes in cystometric parameters such as voiding threshold pressure and intercontraction interval were completely inhibited (Fig. 3).

Next, in the bladder-to-colon cross-sensitization experiments, there were no apparent changes in intracolonic or intravesical pressures after vehicle (saline) injection into the bladder (Fig. 1b). Following the application of AI into the

bladder (intravesically), mean intracolonic pressure was significantly decreased from 30 min after the application (0 min: 6.9 ± 1.6 cmH$_2$O, 30 min: 5.4 ± 1.7 cmH$_2$O, 60 min: 4.3 ± 1.8 cmH$_2$O, 90 min: 3.2 ± 1.7 cmH$_2$O, and 120 min: 2.3 ± 1.8 cmH$_2$O), although mean intravesical pressure was significantly increased from 30 min after the AI application (i.e., 0 min: 7.9 ± 2.3 cmH$_2$O, 30 min: 16.5 ± 2.0 cmH$_2$O, 60 min:
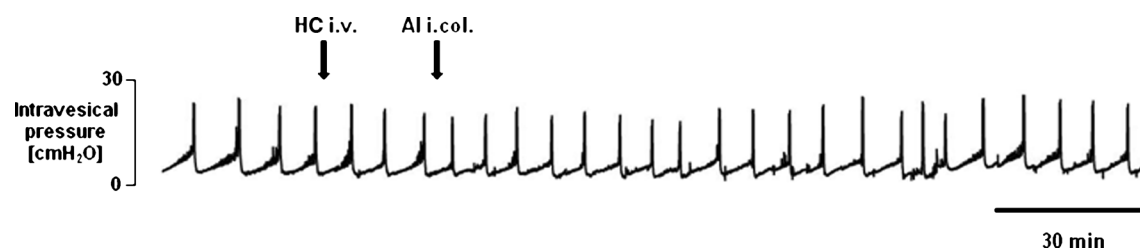
Fig. 3 Representative cystometrograms showing the effects of intracolonic (*i.col.*) allyl isothiocyanate (*AI*), a TRPA1 activator, administration in the presence of HC-030031 (*HC*), a TRPA1 inhibitor, which was intravenously (*i.v.*) applied 30 min before the AI administration.

Bladder overactivity shown by decreases in voiding threshold pressure and intercontraction intervals via TRPA1 stimulation in the colon (Fig. 1) was completely prevented by the pretreatment with HC

Fig. 4 Evans blue (*EB*) dye extravasation, which is an indicator of vascular permeability due to tissue inflammation, 2 h after application of allyl isothiocyanate (*AI*), a TRPA1 activator, into the bladder (**a**) or the colon (**b**). EB dye extravasation was significantly increased in the inflamed organs after the AI application, and also in the bladder after intracolonic AI application (i.e., colon-to-bladder cross-sensitization), but not in the colon after intravesical AI application. $*p < 0.05$ and $**p < 0.01$ compared to the respective vehicle-treated groups
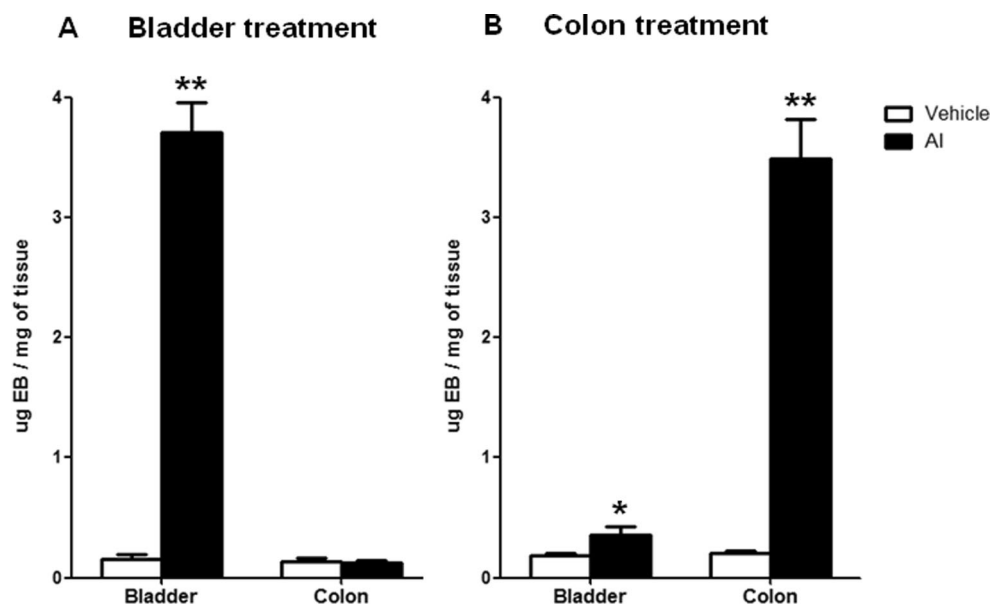
18.9±3.3 cmH$_2$O, 90 min: 19.6±2.4 cmH$_2$O, and 120 min: 19.5±3.0 cmH$_2$O) (Figs. 1b and 2b).

In the rats with AI injection into the colon or the bladder, EB dye extravasation was significantly increased in the inflamed organs (colon: AI 3.5±0.3 μg/mg vs vehicle 0.2±0.1 μg/mg; bladder: AI 3.7±0.3 μg/mg vs vehicle 0.2±0.1 μg/mg) and also in the bladder (AI 0.4± 0.1 μg/mg vs vehicle;0.2±0.1 μg/mg) after intracolonic AI injection (Fig. 4).

Discussion

The results of this study indicate that TRPA1 stimulation in the colon induces colon-to-bladder cross-sensitization, leading to bladder overactivity because intercontraction intervals and voiding threshold pressure during continuous saline infusion into the bladder were significantly decreased and EB dye extravasation was significantly increased in the bladder after intracolonic AI application. On the other hand, TRPA1 channels in the bladder do not seem to induce bladder-to-colon cross-sensitization because TRPA1 stimulation in the bladder elicited bladder overactivity, but not colonic overactivity (Figs. 1 and 2). In addition, it is known that high-dose application of TRPA1 activators to organs can induce desensitization of afferent fibers [21]. However, 50 mM (0.5 %) AI used in the present study is considered not to induce desensitization of colon or bladder afferent pathways, at least during the observation period, because mean intracolonic or intravesical pressures were significantly increased after the AI application into the colon or the bladder, respectively, for up to 120 min.

It has been reported that intracolonic application of TNBS induces a reduction of bladder capacity, which is prevented by the intracolonic pretreatment with resiniferatoxin that induces desensitization of TRPV1-expressing afferents in the colon [13], and that intravesical application of AI induces bladder overactivity, which is blocked by desensitization of capsaicin-sensitive afferent pathways [22]. In this study, we also confirmed that bladder overactivity (i.e., decreased intercontraction intervals and voiding threshold pressure) induced by TRPA1 stimulation in the colon was completely prevented by the i.v. pretreatment with HC, a TRPA1 inhibitor. TRPA1 is selectively expressed by a subset of nociceptive sensory neurons that also express TRPV1 [5], suggesting that TRPV1 and TRPA1 may mutually control the transduction of noxious stimuli in TRPV1-expressing sensory neurons [9, 23]. Furthermore, TRPV1 and TRPA1 are more than just acute damage sensors that mediate acute pain; they also play an important role on inflammatory pain and possibly neuropathic pain [23]. TRPV1 and TRPA1 mediate not only an afferent signal to the dorsal horn of the spinal cord, but also are involved in peripheral efferent function of afferent nerves via secretion of inflammatory agents such as substance P and calcitonin gene-related peptide (CGRP), which causes local neurogenic inflammation [24]. This peripheral inflammation then produces multiple inflammatory mediators such as bradykinin, prostaglandins, purines, proteases, and nerve growth factor that act on their cognate receptors expressed by nociceptors to activate intracellular signal transduction pathways [9]. These pathways can phosphorylate TRPV1 and TRPA1 and thereby alter their trafficking to the membrane and their thresholds and kinetics (peripheral sensitization) [23]. The present study further suggests that TRPA1 channels expressed in capsaicin-sensitive, TRPV1-expressing afferents are significantly involved in the emergence of cross-sensitization among pelvic organs, especially for colon-to bladder sensitization.

There are three different neural cross-talk mechanisms proposed to underlie pelvic organ cross-sensitization [14]. The first mechanism is considered to be an interaction within dorsal root ganglion (DRG) neurons with branching or multiple axons, which could serve as direct neuronal connections among different organ domains. The previous studies using dual retrograde dye labeling in DRG sections have demonstrated that convergent DRG neurons receiving afferent inputs from the distal colon and the bladder are identified in 14–17 % of the total labeled cells [25, 26]. The second mechanism is that afferent inputs from an inflamed structure and from other uninflamed structures converge on the same interneuron located in the dorsal horn of the spinal cord. It has been reported that afferent pathways originating from the bladder and the distal colon send their axons to the similar levels of spinal cord, i.e., the L6–S2 levels through pelvic nerves and the T13–L1 levels through hypogastric nerves [4, 27]. The third mechanism appears to involve higher centers of the brain. It is well known that Barrington's nucleus located at the brain stem is the primary component of the pontine micturition center. The divergent projections of Barrington's nucleus neurons to parasympathetic preganglionic neurons innervating the bladder and the colon have shown that individual neurons within Barrington's nucleus are likely to integrate signals from distal pelvic viscera [28]. Based on these findings, it is assumed that IC/BPS may be induced by central sensitization due to neural cross-talk in the DRG, spinal cord, and/or brain levels.

The present study also demonstrated that TRPA1 stimulation in the colon induced bladder inflammation shown by increased plasma extravasation in the bladder, although plasma extravasation in the colon was not detected after TRPA1 stimulation in the bladder. These results coincide with the findings of colon-to-bladder, but not bladder-to-colon, cross-sensitization in pressure measurement experiments. The exact reason for this one-way sensitization from the colon to the bladder is not known; however, the previous study using mice showed that, in lumbosacral DRG, TRPA1-expressing cells are more numerous in colonic afferent neurons than in bladder afferent neurons [29]. Therefore, TRPA1-mediated pelvic organ cross-sensitization is more likely to occur when the colon is stimulated, rather than when the bladder is stimulated. Furthermore, it has been reported that, when AI was applied into the bladder, colon, or uterus, plasma extravasation was significantly increased in the respective inflamed organs; however, interestingly, either colon or uterus inflammation significantly increased plasma extravasation in the uninflamed bladder, whereas cross-organ sensitizing effects were not seen between the colon and uterus [20]. These findings suggest that the bladder may be the most vulnerable pelvic organ in cases of cross-sensitization in the pelvis, although further studies are needed to clarify this point.

In conclusion, TRPA1 stimulation in the colon can induce bladder overactivity and inflammatory changes, indicative of colon-to-bladder cross-sensitization. These findings suggest that TRPA1 stimulation plays a significant role in pelvic organ cross-sensitization and may contribute to the pathogenesis of IC/BPS, which often overlaps with other chronic pelvic pain conditions.

References

1. Alagiri M, Chottiner S, Ratner V, Slade D, Hanno PM (1997) Interstitial cystitis: unexplained associations with other chronic disease and pain syndromes. Urology 49:52–57
2. Novi JM, Jeronis S, Srinivas S, Srinivasan R, Morgan MA, Arya LA (2005) Risk of irritable bowel syndrome and depression in women with interstitial cystitis: a case-control study. J Urol 174:937–940
3. Blanchard EB, Keefer L, Lackner JM, Galovski TE, Krasner S, Sykes MA (2004) The role of childhood abuse in Axis I and Axis II psychiatric disorders and medical disorders of unknown origin among irritable bowel syndrome patients. J Psychosom Res 56:431–436
4. Berkley KJ (2005) A life of pelvic pain. Physiol Behav 86:272–280
5. Story GM, Peier AM, Reeve AJ et al (2003) ANKTM1, a TRP-like channel expressed in nociceptive neurons, is activated by cold temperatures. Cell 112:819–829
6. Suzuki M, Watanabe Y, Oyama Y et al (2003) Localization of mechanosensitive channel TRPV4 in mouse skin. Neurosci Lett 353:189–192
7. Tsukimi Y, Mizuyachi K, Yamasaki T, Niki T, Hayashi F (2005) Cold response of the bladder in guinea pig: involvement of transient receptor potential channel, TRPM8. Urology 65:406–410
8. Andersson KE, Gratzke C, Hedlund P (2010) The role of the transient receptor potential (TRP) superfamily of cation-selective channels in the management of the overactive bladder. BJU Int 106:1114–1127
9. Patapoutian A, Tate S, Woolf CJ (2009) Transient receptor potential channels: targeting pain at the source. Nat Rev Drug Discov 8:55–68
10. Yamada T, Ugawa S, Ueda T, Ishida Y, Kajita K, Shimada S (2009) Differential localizations of the transient receptor potential channels TRPV4 and TRPV1 in the mouse urinary bladder. J Histochem Cytochem 57:277–287
11. Liu L, Mansfield KJ, Kristiana I, Vaux KJ, Millard RJ, Burcher E (2007) The molecular basis of urgency: regional difference of vanilloid receptor expression in the human urinary bladder. Neurourol Urodyn 26:433–438
12. Akbar A, Yiangou Y, Facer P, Walters JR, Anand P, Ghosh S (2008) Increased capsaicin receptor TRPV1-expressing sensory fibres in irritable bowel syndrome and their correlation with abdominal pain. Gut 57:923–929
13. Asfaw TS, Hypolite J, Northington GM, Arya LA, Wein AJ, Malykhina AP (2011) Acute colonic inflammation triggers detrusor instability via activation of TRPV1 receptors in a rat model of pelvic organ cross-sensitization. Am J Physiol Regul Integr Comp Physiol 300:R1392–R1400
14. Malykhina AP (2007) Neural mechanisms of pelvic organ cross-sensitization. Neuroscience 149:660–672
15. Jordt SE, Bautista DM, Chuang HH et al (2004) Mustard oils and cannabinoids excite sensory nerve fibres through the TRP channel ANKTM1. Nature 427:260–265

16. Brierley SM, Hughes PA, Page AJ et al (2009) The ion channel TRPA1 is required for normal mechanosensation and is modulated by algesic stimuli. Gastroenterology 137:2084–2095

17. Bautista DM, Jordt SE, Nikai T et al (2006) TRPA1 mediates the inflammatory actions of environmental irritants and proalgesic agents. Cell 124:1269–1282

18. Satoh J, Yamakage M (2009) Desflurane induces airway contraction mainly by activating transient receptor potential A1 of sensory C-fibers. J Anesth 23:620–623

19. Peng HY, Chen GD, Tung KC et al (2009) Colon mustard oil instillation induced cross-organ reflex sensitization on the pelvic-urethra reflex activity in rats. Pain 142:75–88

20. Winnard KP, Dmitrieva N, Berkley KJ (2006) Cross-organ interactions between reproductive, gastrointestinal, and urinary tracts: modulation by estrous stage and involvement of the hypogastric nerve. Am J Physiol Regul Integr Comp Physiol 291:R1592–R1601

21. Andrade EL, Ferreira J, André E, Calixto JB (2006) Contractile mechanisms coupled to TRPA1 receptor activation in rat urinary bladder. Biochem Pharmacol 72:104–114

22. Du S, Araki I, Kobayashi H, Zakoji H, Sawada N, Takeda M (2008) Differential expression profile of cold (TRPA1) and cool (TRPM8) receptors in human urogenital organs. Urology 72:450–455

23. Furuta A, Suzuki Y, Hayashi N, Egawa S, Yoshimura N (2012) Transient receptor potential A1 receptor-mediated neural cross-talk and afferent sensitization induced by oxidative stress: implication for the pathogenesis of interstitial cystitis/bladder pain syndrome. Int J Urol 19:429–436

24. Julius D, Basbaum AI (2001) Molecular mechanisms of nociception. Nature 413:203–210

25. Malykhina AP, Qin C, Greenwood-van Meerveld B, Foreman RD, Lupu F, Akbarali HI (2006) Hyperexcitability of convergent colon and bladder dorsal root ganglion neurons after colonic inflammation: mechanism for pelvic organ cross-talk. Neurogastroenterol Motil 18:936–948

26. Christianson JA, Liang R, Ustinova EE, Davis BM, Fraser MO, Pezzone MA (2007) Convergence of bladder and colon sensory innervation occurs at the primary afferent level. Pain 128:235–243

27. de Groat WC (2006) Integrative control of the lower urinary tract: preclinical perspective. Br J Pharmacol 147(Suppl 2):S25–S40

28. Rouzade-Dominguez ML, Miselis R, Valentino RJ (2003) Central representation of bladder and colon revealed by dual transsynaptic tracing in the rat: substrates for pelvic visceral coordination. Eur J Neurosci 18:3311–3324

29. La JH, Schwartz ES, Gebhart GF (2011) Differences in the expression of transient receptor potential channel V1, transient receptor potential channel A1 and mechanosensitive two pore-domain K+ channels between the lumbar splanchnic and pelvic nerve innervations of mouse urinary bladder and colon. Neuroscience 186:179–187

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Title: **Pelvic Organ Cross-sensitization to Enhance Bladder and Urethral**

Pain Behaviors in Rats with Experimental Colitis

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ABSTRACT

Neural cross-sensitization has been postulated as a mechanism underlying

overlaps of chronic pelvic pain disorders such as bladder pain syndrome/interstitial

cystitis (BPS/IC) and irritable bowel syndrome (IBS).   Animals with experimental

colitis have been used to study the underlying mechanisms for overlapped pelvic

pain symptoms, and shown to exhibit bladder overactivity evidenced by frequent

voiding; however, it has not directly been evaluated whether pain sensation derived

from the lower urinary tract is enhanced in colitis models.   Also, the

cross-sensitization between the colon and urethra has not been studied previously.

In the present study, we therefore investigated pain behaviors induced by

nociceptive stimuli in the lower urinary tract and the involvement of C-fiber afferent

pathways using rats with colitis induced by intracolonic application of

2,4,6-trinitrobenzenesulfonic acid (TNBS).   In TNBS-induced colitis rats at 10 days,

intravesical application of resiniferatoxin (RTx) induced a significantly greater

number of episodes of both licking and freezing behaviors, which were reduced by

capsaicin-sensitive C-fiber afferent desensitization. Histochemical studies using

fluorescent dye tracers injected into the colon, bladder or urethra showed that

dichotomized afferent neurons comprised 6.9-14.5% of L1, L6 and S1 dorsal root

ganglion (DRG) neurons innervating the colon or the lower urinary tract.   TRPV1

mRNA expression was significantly increased in, the bladder, urethra and S1 DRG

in colitis rats.   An increase in myeloperoxidase (MPO) activity was found in the

colon, but not in the bladder or urethra after intracolonic TNBS treatment. These

results indicate that TNBS-induced colitis increased pain sensitivity in the bladder

and urethra via activation of C-fiber afferent pathways due to colon-to-bladder and

colon-to-urethral cross-sensitization, suggesting the contribution of pelvic organ

cross-sensitization mechanisms to overlapped pain symptoms in BPS/IC and IBS.

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Introduction

Bladder pain syndrome/interstitial cystitis (BPS/IC) is a chronic urological disorder

characterized as pelvic pain related to bladder filling, coupled with additional

symptoms, such as increased urinary frequency and urgency, without proven

urinary infection or other obvious pathology (Hanno et al., 2011). It is currently

estimated that 3.3 to 7.9 million United States women 18 years old or older is

suffering from BPS/IC (Berry et al., 2011). It has also been reported that over

one-third of patients diagnosed with BPS/IC exhibit symptoms consistent with

irritable bowel syndrome (IBS) (Alagiri et al., 1997, Novi et al., 2005), while 26–56%

of patients diagnosed with IBS also have symptoms of BPS/IC (Maxton et al., 1989,

Blanchard et al., 2004). In addition, BPS/IC patients often report pain in different

and/or additional sites such as the urethra (Warren et al., 2008). However, the

mechanisms underlying the pelvic organ cross-talk that contributes to overlapped

symptoms in chronic pelvic pain syndromes such as BPS/IC, urethral pain and IBS

have not been well clarified.

Previous animal studies demonstrated that TNBS-induced experimental colitis produced lower urinary tract dysfunction such as increased voiding frequency (Liang et al., 2007, Ustinova et al., 2007) and an increase in the firing rate of bladder afferent nerves in response to urinary bladder distension in rats (Ustinova et al., 2007). These animals showed increased expression of neuropeptides such as substance P and calcitonin gene-related peptide (Pan et al., 2010), growth factors and mast cells (Liang et al., 2007) in the bladder. Moreover, a recent report demonstrated that desensitization of the transient receptor potential vanilloid 1 (TRPV1) by intravesical application of resiniferatoxin (RTx) suppressed the increased excitability of bladder spinal neurons in rats with acute colitis induced by intracolonic instillation with 2,4,6-trinitrobenzenesulfonic acid (Malykhina et al., 2013). These results suggest that intracolonic irritation can sensitize bladder afferent pathways, resulting in bladder overactivity. Also, in previous studies, pelvic pain conditions in the colitis model were assessed by the visceromotor response elicited by colorectal distension (Greenwood-Van Meerveld et al., 2005)

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and referred somatic hyperalgesia in the paw and/or abdominal skin regions (Cameron et al., 2008, Claudino et al., 2010). Thus, although the rat with experimental colitis has been used as an animal model of chronic pelvic pain, pain sensation derived from the bladder has not directly been evaluated in experimental colitis. In addition, the cross-talk between the colon and urethra to induce urethral pain has not been studied previously in the colitis model. Since our laboratory developed a rat model that can be used to investigate pain sensation from the bladder and urethra under the freely moving condition by monitoring pain behaviors such as freezing and licking (Saitoh et al., 2008), we investigated whether nociceptive behaviors induced by chemical stimuli in the lower urinary tract are enhanced in colitis rats. We also investigated the number of dichotomized afferent neurons that innervate both colon and bladder or both colon and urethra; as well as changes in gene expression of TRPV1 channels in dorsal root ganglia (DRG), bladder and urethral tissues.

EXPERIMENTAL PROCEDURES

Animals

Sixty-four female Sprague-Dawley rats (206 to 268 g) were used in this study.
Rats were divided into the following groups: (1) 20 rats for behavioral testing, (2) 24
rats for mRNA measurement with RT-PCR, (3) 8 rats for a histochemical study with
fluorescent dye tracers, and (4) 12 rats for MPO activity assay. We used female
rats because of the higher prevalence of BPS/IC and IBS in women than in men
(Clemens et al., 2007, Ito et al., 2007, Hall et al., 2008, Lovell and Ford, 2012) and
the technical easiness in urethral catheterization during behavioral studies in female
rats compared to male rats. All experiments were conducted in accordance with
institutional guidelines and approved by the University of Pittsburgh Institutional
Animal Care and Use Committee.

Induction of experimental colitis

Experimental colitis was induced as described in a previous report (Liang et al.,

2007) with slight modification in the injection volume. Briefly, rats fasted for 24

hours were anesthetized with isoflurane. A polyethylene catheter (PE90) was

inserted from the anus and placed approximately 6 cm proximal to the anal verge,

and then 2,4,6-trinitrobenzenesulfonic acid (TNBS; 50 mg/mL in 50% ethanol, 0.4

mL) was administered through the catheter and retained in the distal colon with the

rats in a vertical position for several minutes. Thereafter, surgilube® (E. Fougera

& Co., Melville, NY) was inserted into the anal canal to prevent the leakage of TNBS

and then rats were returned to the housing facility after the recovery from

anesthesia until each assay. Control animals received the vehicle treatment with

0.4 mL of 50% ethanol. In some rats used for behavioral testing, capsaicin (total

125 mg/kg) was given subcutaneously in divided doses on 2 consecutive days: 25

and 50 mg/kg at a 12-h interval on the first day and 50 mg/kg on the second day, to

induce desensitization of capsaicin-sensitive C-fiber afferent pathways as described

in previous reports (Cheng and de Groat, 2004, Kullmann et al., 2008).

Nociceptive behavior study

The measurement of nociceptive behaviors was conducted according to the previously reported method (Saitoh et al., 2008). In brief, rats were acclimated in metabolic cages (Nalgene Co., Rochester, New York) for three hours, and then placed in a Bollman-type restraining device (KN-326; Natsume Seisakusho, Tokyo, Japan). A polyethylene tube (PE-50; Clay Adams Division of Becton Dickinson, Parsippany, NJ) was inserted into the bladder through the urethra, and residual urine was withdrawn. Thereafter, RTx (0.3 μM), or the corresponding vehicle alone (10% ethanol, 10% Tween 80, and 80% physiological saline), was instilled into the bladder via the catheter at a volume of 0.3 mL and kept for 1 min. The transurethral catheter was then removed and rats were placed back into metabolic cages. Two types of behaviors, licking (lower abdominal licking) and freezing (motionless head-turning towards the lower abdomen), were scored for a 15 min-interval that was divided into 5-sec intervals. When licking or freezing occurred during each 5-sec interval, it was scored as one positive event. The

number of licking or freezing behavior events was summed for each of 5-minute

periods (0 to 5, 5 to 10 and 10 to 15 minutes) following the RTx treatment.   The

intravesical application of the solution (RTx or vehicle) was conducted in a blinded

manner for assessors of animal behaviors.

Retrograde labeling of colon, bladder and urethral afferent neurons

Under isoflurane anesthesia, rats underwent a midline laparotomy to gain access to

the pelvic organs.   The distal colon (2.5-3.5 cm from the rectum) and the bladder or

urethra were exposed, and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine

perchlorate (DiI, Invitrogen Inc., Paisley, UK; 1% w/v in methanol) was injected to

6-8 different sites of the colonic wall using a 30G needle syringe.   Additionally, Fast

Blue (Polysciences Inc., Warrington, PA, USA; 1% w/v in water) was injected into

4-5 different sites of the bladder or urethral wall in order to examine the presence of

DRG neurons innervating the colon and bladder or the colon and urethra,

respectively.   The total volume of dye injected into each organ was 25 μL.   To

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prevent leakage and labeling of adjacent tissues, the needle was left in place for 30

s after injection, and then a cotton swab was applied to prevent leaking.

Abdominal incisions were closed with sutures and rats were returned to housing

facility until each assay.   The rats were post-operatively treated with ampicillin (100

mg/kg, subcutaneously; Fort Dodge Animal Health, Fort Dodge, IA) and

buprenorphine (0.05 mg/kg subcutaneously; Reckitt Benckiser Pharmaceuticals,

Richmond, VA) twice a day for 3 days.

Histological assessment of labeled DRG cells

Ten days after the injection of dyes, rats were euthanized with pentobarbital, and

DRG at Th13 to S2 levels were removed bilaterally.   The specimens were

embedded into OTC compound, frozen with dry ice/isopentane (2-methylbutane)

and stored at -80 °C until used.   Serial transverse sections were cut at 10 μm

thickness with a cryostat, and every fourth sections were mounted on slides to avoid

duplicate counting of the cells.   Positively labeled cells were evaluated with an

Olympus fluorescence microscope with a multiband filter set for DiI and FB. Three

sections each from the right and left DRG at each level were randomly selected in a

rat, and the number of dye-labeled cells per section was counted. Then, the

number of dye-labelled cells in 6 sections per one DRG level was averaged in each

rat, and the data was presented as mean ± SEM of 4 rats. The percentage of dual

labeled neurons was determined as a ratio against the sum of DiI or FB labeled

neurons (Malykhina et al., 2006, Christianson et al., 2007).

Myeloperoxidase (MPO) activity assay

Myeloperoxidase is an enzyme fond primarily contained in neutrophils; and the

measurement has been used as a quantitative index of inflammation in tissues

including the colon from rats with colitis (Smith and Castro, 1978, Morris et al., 1989,

Yang et al., 2008). Colon, bladder and urethral tissues were rinsed with cold PBS

(pH 7.4), weighed, and were homogenized for 30 s at 4℃ after addition of 200 μl

RIPA lysis buffer (sc-24948; Santa Cruz biotechnology, California) per 10 mg of

tissue and were homogenized for 30 s at 4℃.   The homogenates were incubated

for 30 min on ice, and then centrifuged at 10,000 g for 10 min at 4°C.   The levels of

MPO in the supernatants were measured by using a MPO assay ELISA Kit (HK105;

Hycult biotechnology, Netherlands) according to the manufacturer's instruction.

Quantification was performed by measuring absorbance at 450 nm using a

microplate reader.   The protein concentrations of supernatants were also

measured by using BCA Protein Assay Kit (#23225; Pierce Biotechnology,

Rockford) according to the manufacturer's instruction.   The MPO concentration

was standardized relative to protein levels and expressed in ng/mg total protein.

RNA isolation and real-time PCR analysis

One μg of total RNA extracted from L6-S1 DRG, bladder or urethra tissues was

reverse-transcribed into cDNA using ThermoScript^{TM} RT-PCR system (Invitrogen,

Carlsbad, CA) according to the manufacturer's instruction.   Quantitative PCR was

performed with an MX3000P real-time PCR system (Stratagene, La Jolla, CA) in a

25 μL volume using SYB Green PCR Master Mix (QIAGEN, Velencia, CA). cDNA

product was amplified by 40 cycles (denaturation at 95°C for 15 sec; primer

annealing for 55°C for 60 sec; and elongation for 72°C for 30 sec).   Primers

sequences used for real-time PCR were the following: 5'- AGT AAC TGC CAG

GAG CTG GA-3' (forward) and 5'- GTG TCA TTC TGC CCA TTG TG-3' (reverse)

for rat TRPV1 and 5'- GGC CAA AAG GGT CAT CAT CT-3' (forward) and 5'- GTG

ATG GCA TGG ACT GTG GT-3' (reverse) for rat GAPDH, which is a house

keeping gene used as the internal control.   All primers for PCR reaction were

designed based on the NCBI database sequence of rat reference mRNA and

checked for specificity with BLAST software from the NCBI website; and PCR

products were validated by size determination after separation on 2% agarose gel.

Drugs

2,4,6-Trinitrobenzenesulfonic acid (TNBS), capsaicin and resiniferatoxin (RTx) were

purchased from Sigma-Aldrich (St Louis, MO).   Capsaicin and RTx were dissolved

in a vehicle consisting of 10% ethanol, 10% Tween 80, and 80% physiological

saline.

Statistical analysis

All data were represented as the mean ± SEM. Statistical significance was

determined by unpaired t-test (two-tailed) or one-way ANOVA followed by

Bonferroni's Multiple Comparison test. The P values < 0.05 were considered

significant.

RESULTS

Nociceptive behaviors in conscious rats treated with TNBS

At day 10 after intracolonic administration of TNBS, rats did not show apparent pain

behaviors such as freezing or licking compared with vehicle (for TNBS)-treated

control rats when noxious stimulation was not applied (Fig. 1; intravesical

application of vehicle for RTx). When RTx (0.3 μM) was intravesically applied,

TNBS-untreated control rats exhibited no apparent freezing behavior with slightly

enhanced licking behavior only for the initial 5-minute observation period. On the

other hand, a significantly greater number of episodes of both licking and freezing

behaviors were shown after intravesical application of RTx (0.3 μM) in rats with

TNBS-induced colitis compared with the vehicle-treated group (Figs. 2C, 2D; $P <$

0.001, respectively), indicating increased pain sensitivity to noxious stimuli in the

lower urinary tract following TNBS-induced colitis. In addition, these enhanced

pain behaviors were significantly reduced with pre-treatment with capsaicin that

induced desensitization of capsaicin-sensitive C-fiber afferent pathways (Figs. 2C,

2D; P < 0.01, respectively).

Myeloperoxidase (MPO) activity

At day 10 after intracolonic application of TNBS, MPO activity in the colon was

significantly (P < 0.01) increased compared with the vehicle treated group (Fig. 3).

On the other hand, there was no change in MPO activities in either the bladder or

urethra in rats with TNBS-induced colitis (Fig. 3).

Distribution of afferent neurons innervating colon, bladder and urethra

DRG neurons retrogradely labeled by fluorescent dyes injected into the bladder,

urethra or colon are counted in DRG sections at T13 to S2 levels.   Colonic afferent

neurons labeled with DiI were distributed in L1, L2, L6 and S1 DRGs with abundant

labeling in L1 and S1 DRG neurons whereas bladder or urethra afferent neurons

labeled by FB injected into the bladder or urethra, respectively, were distributed in

L1, L6 and S1 DRGs with abundant labeling in L6 DRG neurons (Fig. 5).   In T13,

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L3-L5 and S2 DRGs, few or no cells were labeled with either FB or DiI.

Dichotomized afferent neurons innervating both colon and bladder or both colon

and urethra were observed in L1, L6 and S1 DRGs (Figs. 4 and 5).   When DiI and

FB were injected into the colon and bladder, respectively, the ratio of afferent

neurons with dichotomizing projection to both colon and bladder was 6.48-14.3%

among colon or bladder-innervating afferent neurons; specifically 6.48 ± 2.26 % in

L1 DRG, 14.0 ± 2.50 % in L6 DRG and 14.3 ± 1.99 % in S1 DRG expressed as

percentage of DiI and FB dually-labeled neurons among the sum of DiI or FB

labeled neurons in the colon/bladder dye-injected group (n=4 rats).   In another

series of experiments, when DiI and FB were injected into the colon and urethra,

respectively, the ratio of afferent neurons with dichotomizing projection to the colon

and urethra was 7.41-9.30% among colon or urethra-innervating afferent neurons;

specifically 7.41 ± 1.78 % in L1 DRG,, 9.30 ± 0.53 % in L6 DRG and 8.43 ± 1.85 %

in S1 DRG, expressed as percentage of DiI and FB dually-labeled neurons among

the sum of DiI or FB labeled neurons in the colon/urethra dye-injected group (n=4

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rats).

Changes in TRPV1 mRNA in the DRG, bladder and urethra after TNBS treatment in rats

In TNBS-treated colitis rats, the mRNA levels of TRPV1 in the bladder, urethra and S1 DRG were significantly increased compared to sham rats (Figs. 6A, 6B, 6D; $P < 0.05$, respectively).

DISCUSSION

The results in the present study demonstrated that experimental colitis dramatically

enhanced the freezing behavior induced by intravesically applied RTx (TRPV1

receptor agonist) at a low concentration (0.3 μM), which induced only a few events

of nociceptive behaviors in TNBS-untreated control rats. Freezing behavior

induced by intravesical application of RTx in rats was characterized as a typical

nociceptive response to activation of pelvic nerve afferents innervating the bladder

(Saitoh et al., 2008). Also, our previous studies showed that the delivery of

therapeutic genes encoding enkephalins or anti-inflammatory cytokines to the

bladder and bladder afferent pathways using non-replicating herpes simplex virus

vectors is effective to reduce freezing behavior induced by intravesical RTx

administration without affecting licking behavior in rats (Funahashi et al., 2013,

Oguchi et al., 2013, Yokoyama et al., 2013), indicating that RTx-induced freezing

behavior represents the pain behavior derived from nociceptive stimulation of the

bladder. Although previous studies demonstrated that rats with colitis induced by

TNBS have frequent micturition and increased afferent excitability in response to

urinary bladder distention in anesthetized rats (Ustinova et al., 2007), increased

bladder pain sensitivity in this colitis model had not yet been shown. In addition,

von Frey testing in the paw and/or abdominal skin regions has been used to

evaluate the referred pelvic pain in rodent colitis models (Cameron et al., 2008,

Claudino et al., 2010); however, it is not known how the referred somatic

hyperalgesia in these regions correlates to pain sensation derived from the bladder.

In this regard, our current results directly demonstrated that TNBS-induced colitis at

10 days enhances bladder pain sensation elicited by nociceptive stimuli in the

bladder (i.e., freezing behavior) in rats. Furthermore, we confirmed that

dichotomized afferent neurons projecting to both the distal colon and bladder are

identified in DRGs at the L1, L6 and S1 levels, the percentage of which (6.5-14.3%)

is in accordance with the results in the previous reports (Malykhina et al., 2006,

Christianson et al., 2007), supporting the previous notion that the existence of

dichotomizing pelvic afferents could provide a pre-existing neuronal network for

potential pelvic organ cross-sensitization (Ustinova et al., 2010).

Another new finding in this study is an existence of an organ cross-talk between the colon and urethra following TNBS-induced colitis because the cross-talk between these two organs has not been evaluated previously. The present study showed that dichotomization of sensory neurons projecting to the distal colon and urethra were shown at L1, L6 and S1 levels (7.4-9.3 % of the sum of colon or urethral afferent neurons) in rats and that colitis rats showed significantly enhanced licking behavior after the intravesical application of RTx. Because the licking behavior induced after intravesical application of RTx was prevented by transection of the pudendal nerves that innervate the urethra, but not the bladder (Saitoh et al., 2008), it is assumed that RTx-induced licking behavior represents urethral pain due to afferent nerve activation in the pudendal nerve by RTx expelled from the bladder to the urethra during voiding. Therefore, these data suggest that the existence of dichotomizing pelvic afferents between the colon and urethra could contribute to pelvic organ cross-sensitization between these two organs to increase

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pain sensitivity in the urethra after colonic inflammation. Increased bladder and

urethral pain sensitivity after colonic inflammation in this study may explain at least

in part the frequent association of urethral pain in BPS/IC patients (Warren et al.,

2008).

In this study, increased MPO activity, which correlates with the tissue

neutrophil infiltration, was found in the colon, but not in the bladder or urethra,

following TNBS-induced colitis. These results indicate that pain hypersensitivity of

the bladder and urethra in rats with TNBS-induced colon inflammation is produced

by the indirect mechanisms in the lower urinary tract other than inflammatory

changes directly elicited by TNBS administered into the colon. Previous studies

have demonstrated that pelvic organ cross sensitization is induced by activation of

dichotomized afferents innervating different pelvic organs and that activation of

nociceptive C-fiber afferents in one organ could sensitize afferent pathways in

another organ to release neuropeptides such as substance P that trigger

neurogenic inflammation and mast cell activation (Malykhina et al., 2012, Fitzgerald

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et al., 2013).   Also, Pan et al. demonstrated that desensitization of

TRPV1-expressing afferent pathways by intracolonic application of RTx, a TRPV1

receptor agonist, prior to the induction of colonic inflammation prevented the

release of these peptides from the peripheral nerve terminals and reduced the

development of neurogenic cross-talk between the colon and bladder (Pan et al.,

2010).   These results suggest that TRPV1-expressing C-fiber afferent pathways

significantly contribute to the colon-to-bladder cross sensitization following colitis.

Clinically, an increase in TRPV1 expression in the bladder has also been reported in

patients with chronic pelvic pain syndromes including BPS/IC (Lowe et al., 1997, Liu

et al., 2007, Akbar et al., 2008, Poli-Neto et al., 2009).   In addition, previous

preclinical studies demonstrated that immunohistochemical upregulation of TRPV1

in L6-S1 DRG innervating the colon was observed at 72 h after the induction of

colitis with TNBS in rats (De Schepper et al., 2008).   Our results also showed

upregulation of TRPV1 mRNA expression in the bladder, urethra and S1 DRG as

well as enhanced pain behaviors, which was reduced after desensitization of

capsaicin-sensitive afferent pathways in rats with TNBS-induced colitis rats.

These results further support the contribution of TRPV1-expressing C-fiber afferent

pathways to both colon-to-bladder and colon-to-urethra cross sensitizations

induced by colitis, which induce increased pain sensation in the bladder and urethra,

respectively.

Previous studies reported that the combination rat model of endometriosis

and ureteral calculosis shows enhanced pain behavior from both the urinary tract

and female reproductive area, indicating that these two comorbidities strengthen

the viscero-visceral cross-talk (Giamberardino et al., 2002, Lopopolo et al., 2014).

Therefore, future studies using animal models including those of combination

visceral pain could further clarify the underlying mechanisms and help explore new

therapeutic targets for clinical symptoms in chronic pain syndromes.

CONCLUSION

We demonstrated that TNBS-induced colitis at 10 days enhances pain sensation derived from the bladder and urethra as evidenced by increased freezing and licking behaviors elicited by nociceptive bladder and urethral stimuli, respectively. These enhanced pain behaviors are considered to be due to activation of TRPV1-expressing C-fiber afferent pathways, possibly through dichotomized afferents innervating both colon and lower urinary tract. These pelvic organ cross-sensitization mechanisms could be involved in overlapped pain symptoms in BPS/IC and IBS.

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CONFLICT OF INTEREST

None of the authors has any conflict of interest with any of the data presented in this

manuscript.

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REFERENCES

Akbar A, Yiangou Y, Facer P, Walters JR, Anand P, Ghosh S (2008) Increased capsaicin receptor TRPV1-expressing sensory fibres in irritable bowel syndrome and their correlation with abdominal pain. Gut 57:923-929.

Alagiri M, Chottiner S, Ratner V, Slade D, Hanno PM (1997) Interstitial cystitis: unexplained associations with other chronic disease and pain syndromes. Urology 49:52-57.

Berry SH, Elliott MN, Suttorp M, Bogart LM, Stoto MA, Eggers P, Nyberg L, Clemens JQ (2011) Prevalence of symptoms of bladder pain syndrome/interstitial cystitis among adult females in the United States. J Urol 186:540-544.

Blanchard EB, Keefer L, Lackner JM, Galovski TE, Krasner S, Sykes MA (2004) The role of childhood abuse in Axis I and Axis II psychiatric disorders and medical disorders of unknown origin among irritable bowel syndrome patients. J Psychosom Res 56:431-436.

Cameron DM, Brennan TJ, Gebhart GF (2008) Hind paw incision in the rat produces long-lasting colon hypersensitivity. J Pain 9:246-253.

Cheng CL, de Groat WC (2004) The role of capsaicin-sensitive afferent fibers in the lower urinary tract dysfunction induced by chronic spinal cord injury in rats. Exp Neurol 187:445-454.

Christianson JA, Liang R, Ustinova EE, Davis BM, Fraser MO, Pezzone MA (2007) Convergence of bladder and colon sensory innervation occurs at the primary afferent level. Pain 128:235-243.

Claudino RF, Marcon R, Bento AF, Chichorro JG, Rae GA (2010) Endothelins implicated in referred mechanical hyperalgesia associated with colitis induced by TNBS in mice. Can J Physiol Pharmacol 88:661-667.

Clemens JQ, Link CL, Eggers PW, Kusek JW, Nyberg LM, Jr., McKinlay JB (2007) Prevalence of painful bladder symptoms and effect on quality of life in black, Hispanic and white men and women. J Urol 177:1390-1394.

De Schepper HU, De Winter BY, Van Nassauw L, Timmermans JP, Herman AG, Pelckmans

PA, De Man JG (2008) TRPV1 receptors on unmyelinated C-fibres mediate colitis-induced sensitization of pelvic afferent nerve fibres in rats. J Physiol 586:5247-5258.

Fitzgerald JJ, Ustinova E, Koronowski KB, de Groat WC, Pezzone MA (2013) Evidence for the role of mast cells in colon-bladder cross organ sensitization. Auton Neurosci 173:6-13.

Funahashi Y, Oguchi T, Goins WF, Gotoh M, Tyagi P, Goss JR, Glorioso JC, Yoshimura N (2013) Herpes simplex virus vector mediated gene therapy of tumor necrosis factor-alpha blockade for bladder overactivity and nociception in rats. J Urol 189:366-373.

Giamberardino MA, Berkley KJ, Affaitati G, Lerza R, Centurione L, Lapenna D, Vecchiet L (2002) Influence of endometriosis on pain behaviors and muscle hyperalgesia induced by a ureteral calculosis in female rats. Pain 95:247-257.

Greenwood-Van Meerveld B, Johnson AC, Foreman RD, Linderoth B (2005) Spinal cord stimulation attenuates visceromotor reflexes in a rat model of post-inflammatory colonic hypersensitivity. Auton Neurosci 122:69-76.

Hall SA, Link CL, Pulliam SJ, Hanno PM, Eggers PW, Kusek JW, McKinlay JB (2008) The relationship of common medical conditions and medication use with symptoms of painful bladder syndrome: results from the Boston area community health survey. J Urol 180:593-598.

Hanno PM, Burks DA, Clemens JQ, Dmochowski RR, Erickson D, Fitzgerald MP, Forrest JB, Gordon B, Gray M, Mayer RD, Newman D, Nyberg L, Jr., Payne CK, Wesselmann U, Faraday MM (2011) AUA guideline for the diagnosis and treatment of interstitial cystitis/bladder pain syndrome. J Urol 185:2162-2170.

Ito T, Ueda T, Honma Y, Takei M (2007) Recent trends in patient characteristics and therapeutic choices for interstitial cystitis: analysis of 282 Japanese patients. Int J Urol 14:1068-1070.

Kullmann FA, Artim DE, Birder LA, de Groat WC (2008) Activation of muscarinic receptors in rat bladder sensory pathways alters reflex bladder activity. J Neurosci 28:1977-1987.

Liang R, Ustinova EE, Patnam R, Fraser MO, Gutkin DW, Pezzone MA (2007) Enhanced

expression of mast cell growth factor and mast cell activation in the bladder following the resolution of trinitrobenzenesulfonic acid (TNBS) colitis in female rats. Neurourol Urodyn 26:887-893.

Liu L, Mansfield KJ, Kristiana I, Vaux KJ, Millard RJ, Burcher E (2007) The molecular basis of urgency: regional difference of vanilloid receptor expression in the human urinary bladder. Neurourol Urodyn 26:433-438; discussion 439; discussion 451-433.

Lopopolo M, Affaitati G, Fabrizio A, Massimini F, Lapenna D, Giamberardino MA, Costantini R (2014) Effects of tramadol on viscero-visceral hyperalgesia in a rat model of endometriosis plus ureteral calculosis. Fundam Clin Pharmacol 28:331-341.

Lovell RM, Ford AC (2012) Global prevalence of and risk factors for irritable bowel syndrome: a meta-analysis. Clin Gastroenterol Hepatol 10:712-721 e714.

Lowe EM, Anand P, Terenghi G, Williams-Chestnut RE, Sinicropi DV, Osborne JL (1997) Increased nerve growth factor levels in the urinary bladder of women with idiopathic sensory urgency and interstitial cystitis. Br J Urol 79:572-577.

Malykhina AP, Qin C, Greenwood-van Meerveld B, Foreman RD, Lupu F, Akbarali HI (2006) Hyperexcitability of convergent colon and bladder dorsal root ganglion neurons after colonic inflammation: mechanism for pelvic organ cross-talk. Neurogastroenterol Motil 18:936-948.

Malykhina AP, Qin C, Lei Q, Pan XQ, Greenwood-Van Meerveld B, Foreman RD (2013) Differential effects of intravesical resiniferatoxin on excitability of bladder spinal neurons upon colon-bladder cross-sensitization. Brain Res 1491:213-224.

Malykhina AP, Wyndaele JJ, Andersson KE, De Wachter S, Dmochowski RR (2012) Do the urinary bladder and large bowel interact, in sickness or in health? ICI-RS 2011. Neurourol Urodyn 31:352-358.

Maxton DG, Morris JA, Whorwell PJ (1989) Ranking of symptoms by patients with the irritable bowel syndrome. BMJ 299:1138.

Morris GP, Beck PL, Herridge MS, Depew WT, Szewczuk MR, Wallace JL (1989) Hapten-induced model of chronic inflammation and ulceration in the rat colon. Gastroenterology 96:795-803.

Novi JM, Jeronis S, Srinivas S, Srinivasan R, Morgan MA, Arya LA (2005) Risk of irritable bowel syndrome and depression in women with interstitial cystitis: a case-control study. J Urol 174:937-940.

Oguchi T, Funahashi Y, Yokoyama H, Nishizawa O, Goins WF, Goss JR, Glorioso JC, Yoshimura N (2013) Effect of herpes simplex virus vector-mediated interleukin-4 gene therapy on bladder overactivity and nociception. Gene Ther 20:194-200.

Pan XQ, Gonzalez JA, Chang S, Chacko S, Wein AJ, Malykhina AP (2010) Experimental colitis triggers the release of substance P and calcitonin gene-related peptide in the urinary bladder via TRPV1 signaling pathways. Exp Neurol 225:262-273.

Poli-Neto OB, Filho AA, Rosa e Silva JC, Barbosa Hde F, Candido Dos Reis FJ, Nogueira AA (2009) Increased capsaicin receptor TRPV1 in the peritoneum of women with chronic pelvic pain. Clin J Pain 25:218-222.

Saitoh C, Chancellor MB, de Groat WC, Yoshimura N (2008) Effects of intravesical instillation of resiniferatoxin on bladder function and nociceptive behavior in freely moving, conscious rats. J Urol 179:359-364.

Smith JW, Castro GA (1978) Relation of peroxidase activity in gut mucosa to inflammation. Am J Physiol 234:R72-79.

Ustinova EE, Fraser MO, Pezzone MA (2010) Cross-talk and sensitization of bladder afferent nerves. Neurourol Urodyn 29:77-81.

Ustinova EE, Gutkin DW, Pezzone MA (2007) Sensitization of pelvic nerve afferents and mast cell infiltration in the urinary bladder following chronic colonic irritation is mediated by neuropeptides. Am J Physiol Renal Physiol 292:F123-130.

Warren JW, Langenberg P, Greenberg P, Diggs C, Jacobs S, Wesselmann U (2008) Sites of pain from interstitial cystitis/painful bladder syndrome. J Urol 180:1373-1377.

Yang X, Han JQ, Liu R (2008) Effects of experimental colitis on the expressions of calcitonin gene-related peptide and vanilloid receptor 1 in rat spinal cord sensory neurons. Sheng Li Xue Bao 60:143-148.

Yokoyama H, Oguchi T, Goins WF, Goss JR, Nishizawa O, de Groat WC, Wolfe D, Krisky DM, Glorioso JC, Yoshimura N (2013) Effects of herpes simplex virus vector-mediated enkephalin gene therapy on bladder overactivity and nociception. Hum Gene Ther 24:170-180.

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Figure Legends

Fig. 1. Nociceptive behaviors, licking and freezing in rats with intracolonic administration of TNBS.

A and B: Effects of intracolonic administration of TNBS on licking (A) and freezing (B) behavior events in the absence of intravesical noxious stimulation in rats with vehicle (0.4 mL of 50% ethanol) or TNBS injection into the colon. Open and closed circle indicate behavioral events after intravesical application of vehicle (10% ethanol, 10% tween 80 and 80% saline used for dissolving RTx) in rats with intracolonic vehicle and TNBS injection, respectively.

C, D, E and F: Nociceptive behaviors induced by intravesical application of RTx in rats with intracolonic administration of TNBS. TNBS-untreated rats (vehicle for TNBS) with intravesical treatment of vehicle (intravesical vehicle for RTx) or RTx were also included as controls. After RTx (0.3 μM) was applied intravesically for 1 min, the number of licking and freezing behavior events was counted for 15 min, and summed for every 1 minute and plotted in C and D, respectively. The total

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number of licking and freezing behavior events for each of 5-minute periods after

the instillation by RTx is shown in E and F, respectively.　Some TNBS-treated rats

were treated with capsaicin 4 days before the observation (capsaicin-pretreated) to

investigate the effect of desensitization of capsaicin-sensitive afferent pathways.

Each point or bar represents the mean ± SEM from 5 or 6 rats. Vehicle for RTx was

10% ethanol, 10% tween 80 and 80% saline.　Vehicle for TNBS was 50% ethanol.

*P < 0.05; **P < 0.01; **P < 0.001 (two-tailed, unpaired t-test or one-way ANOVA

followed by Bonferroni's Multiple Comparison test).

Fig. 2.　Effects of intracolonic administration of TNBS on myeloperoxidase (MPO)

activities in the colon, bladder and urethra in rats.　MPO activities were quantified

10 days after the induction of colitis in rats.　Each bar represents the mean ± SEM

from 6 rats. Sham was treated with the vehicle for TNBS (50% ethanol).　**P < 0.01

vs. the sham group (two-tailed, unpaired t- test).

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Fig. 3. Representative photomicrographs of DRG sections showing retrogradely

labeled DRG neurons 10 days after the injection of DiI into the colon and FB into the

bladder or urethra. Upper panels (A-C) display the fluorescent image of the same

L6 DRG section showing FB-labeled bladder afferent neurons (A), DiI-labeled colon

afferent neurons (B) and dichotomized afferent neurons innervating both colon and

bladder indicated by yellow arrows (C).   Lower panels (D-F) display the fluorescent

image of the same S1 DRG section showing FB-labeled urethral afferent neurons

(D), DiI-labeled colon afferent neurons (E) and dichotomized afferent neurons

innervating both colon and urethra indicated by yellow arrows (F). Calibration bars:

50 μm.

Fig. 4. Distribution of afferent neurons projected to the colon, bladder and urethra

in dorsal root ganglia at the level of T3 to S2 in rats.   (A) the distribution of labeled

neurons after DiI and FB injection into the distal colon and bladder wall, respectively.

In each bar, colonic, bladder and dually-labeled afferent neurons are expressed as

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shown in panel A.   (B) the distribution of labeled neurons after DiI and FB injection

into the distal colon and urethral wall, respectively. In each bar, colonic, urethral and

dually-labeled afferent neurons are expressed as in panel B.   At each DRG level,

the number of labeled neurons was averaged in 6 sections (3 each from light or left

side) in one rat; then the data was expressed as mean ± SEM of 4 rats.

Fig. 5.   Expression levels of TRPV1 mRNA against a house keeping gene

(GAPDH) in the bladder (A), urethra (B), L6 DRG (C) and S1 DRG (D) in vehicle

(Sham) and TNBS-treated rats.

*$P < 0.05$ vs. the sham group (two-tailed, unpaired *t*-test).

Fig. 1.

A

B

C

D

Fig. 1. (continued)

Figure 2
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Fig. 2.

Figure 3
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Fig. 3.

Figure 4
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Fig. 4.

Figure 5
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Fig. 5.

A

Bladder

B

Urethra

C

L6 DRG

D

S1 DRG

Differential contribution of Kv4-containing channels to A-type, voltage-gated potassium currents in somatic and visceral dorsal root ganglion neurons

AQ:1-2
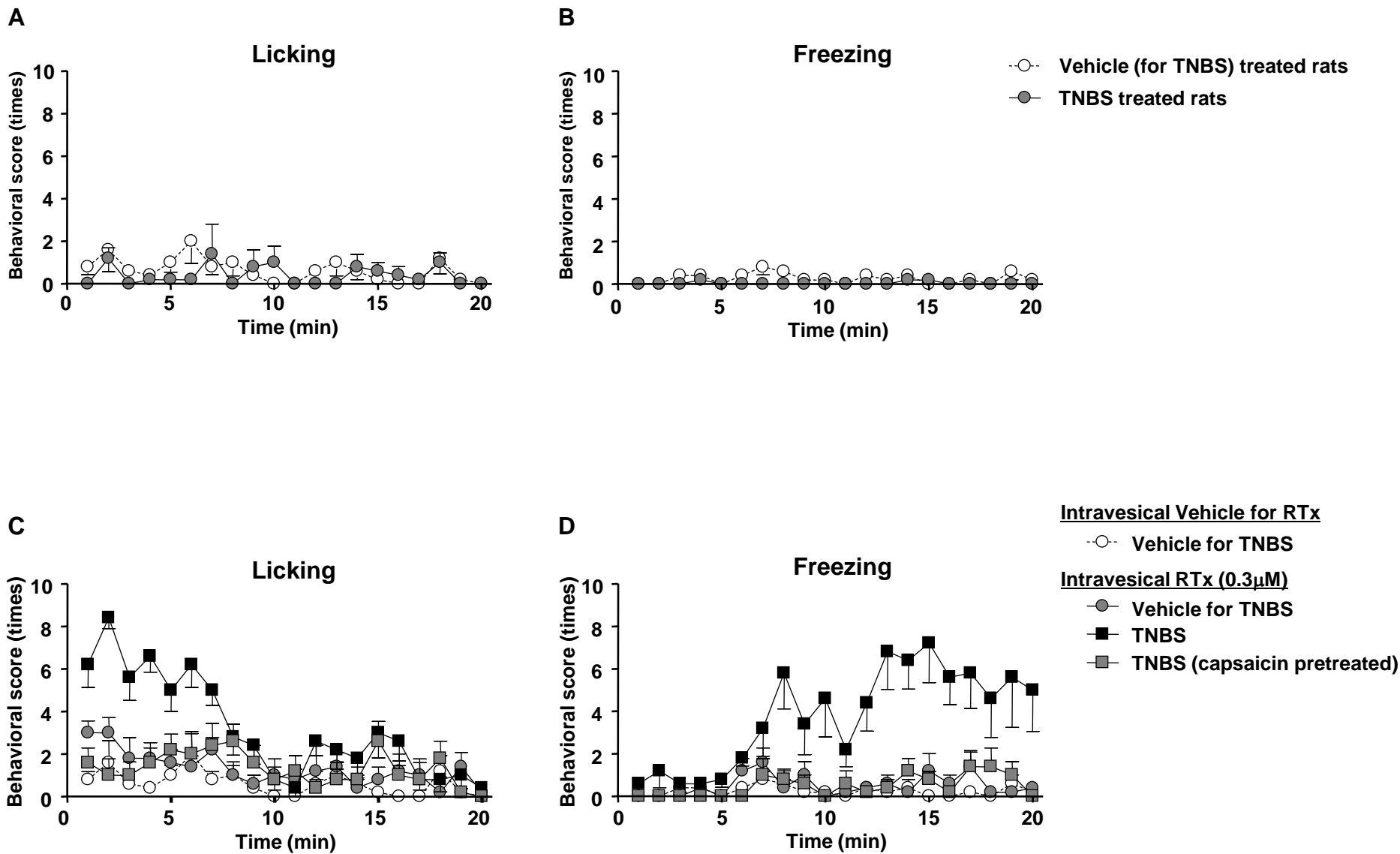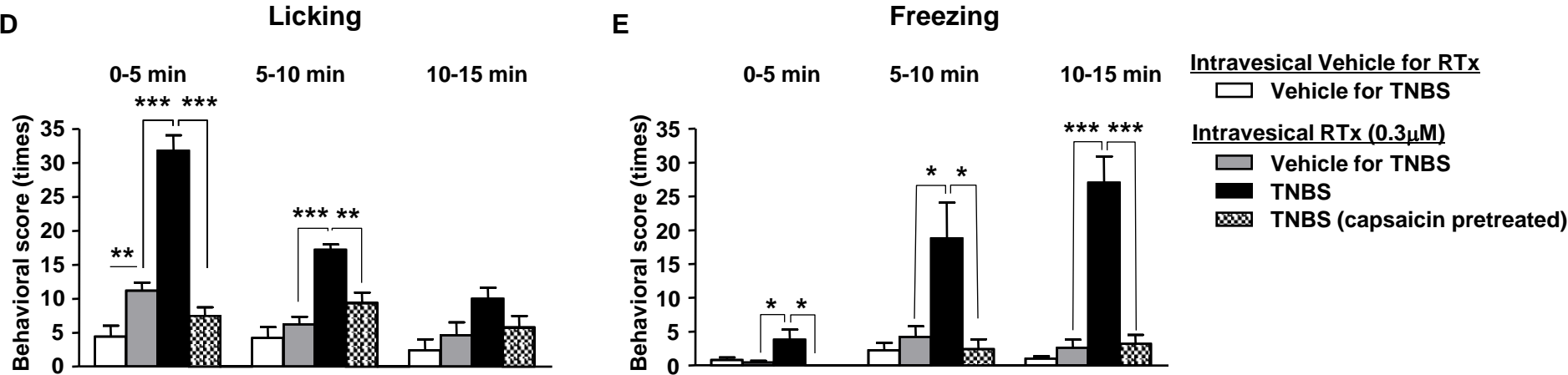
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[1]*Department of Urology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania;* [2]*Department of Environmental and Occupational Health, University of Pittsburgh Graduate School of Public Health, Pittsburgh, Pennsylvania;* [3]*Department of Urology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan; and* [4]*Department of Bioengineering, Nagaoka University of Technology, Nagaoka, Japan*

AQ:3

Yunoki T, Takimoto K, Kita K, Funahashi Y, Takahashi R, Matsuyoshi H, Naito S, Yoshimura N. Differential contribution of Kv4-containing channels to A-type, voltage-gated potassium currents in somatic and visceral dorsal root ganglion neurons. *J Neurophysiol* 112: 000–000, 2014. First published August 20, 2014; doi:10.1152/jn.00054.2014.—Little is known about electrophysiological differences of A-type transient K^+ (K_A) currents in nociceptive afferent neurons that innervate somatic and visceral tissues. Staining with isolectin B4 (IB4)-FITC classifies L6-S1 dorsal root ganglion (DRG) neurons into three populations with distinct staining intensities: negative to weak, moderate, and intense fluorescence signals. All IB4 intensely stained cells are negative for a fluorescent dye, Fast Blue (FB), injected into the bladder wall, whereas a fraction of somatic neurons labeled by FB, injected to the external urethral dermis, is intensely stained with IB4. In whole-cell, patch-clamp recordings, phrixotoxin 2 (PaTx2), a voltage-gated K^+ (Kv)4 channel blocker, exhibits voltage-independent inhibition of the K_A current in IB4 intensely stained cells but not the one in bladder-innervating cells. The toxin also shows voltage-independent inhibition of heterologously expressed Kv4.1 current, whereas its inhibition of Kv4.2 and Kv4.3 currents is voltage dependent. The swapping of four amino acids at the carboxyl portion of the S3 region between Kv4.1 and Kv4.2 transfers this characteristic. RT-PCRs detected Kv4.1 and the long isoform of Kv4.3 mRNAs without significant Kv4.2 mRNA in L6-S1 DRGs. Kv4.1 and Kv4.3 mRNA levels were higher in laser-captured, IB4-stained than in bladder afferent neurons. These results indicate that PaTx2 acts differently on channels in the Kv4 family and that Kv4.1 and possibly Kv4.3 subunits functionally participate in the formation of K_A channels in a subpopulation of somatic C-fiber neurons but not in visceral C-fiber neurons innervating the bladder.

A-type K channel; dorsal root ganglion; nociceptive C-fiber; phrixotoxin 2; Kv4 channel

PRIMARY AFFERENT NEURONS RESPONSIBLE for pain sensation contain lightly myelinated or unmyelinated fibers with medium- to small-sized cell bodies. Neurons with unmyelinated fibers (C-fiber neurons) are responsible for chronic and intractable pain with clinical significance. Neurochemically, C-fiber neurons are divided further into two major subtypes. One group contains neuropeptides, such as calcitonin gene-related peptide (CGRP) and substance P, whereas the other group lacks peptides but contains fluoride-resistant acid phosphatase activity and binds to the *Griffonia simplicifolia* isolectin B4 (IB4) (Nagy and Hunt 1982; Silverman and Kruger 1990). Although the presence of two C-fiber neuron types is well established, their differences in innervating tissues remain uncertain. For example, retrograde labeling indica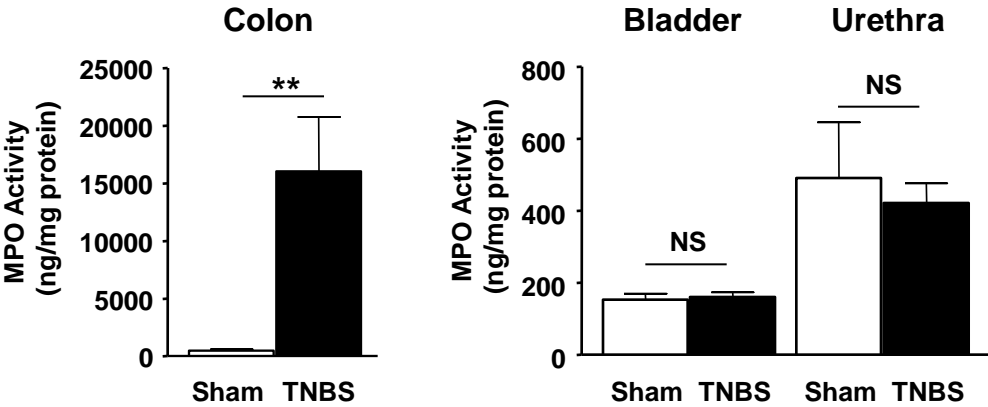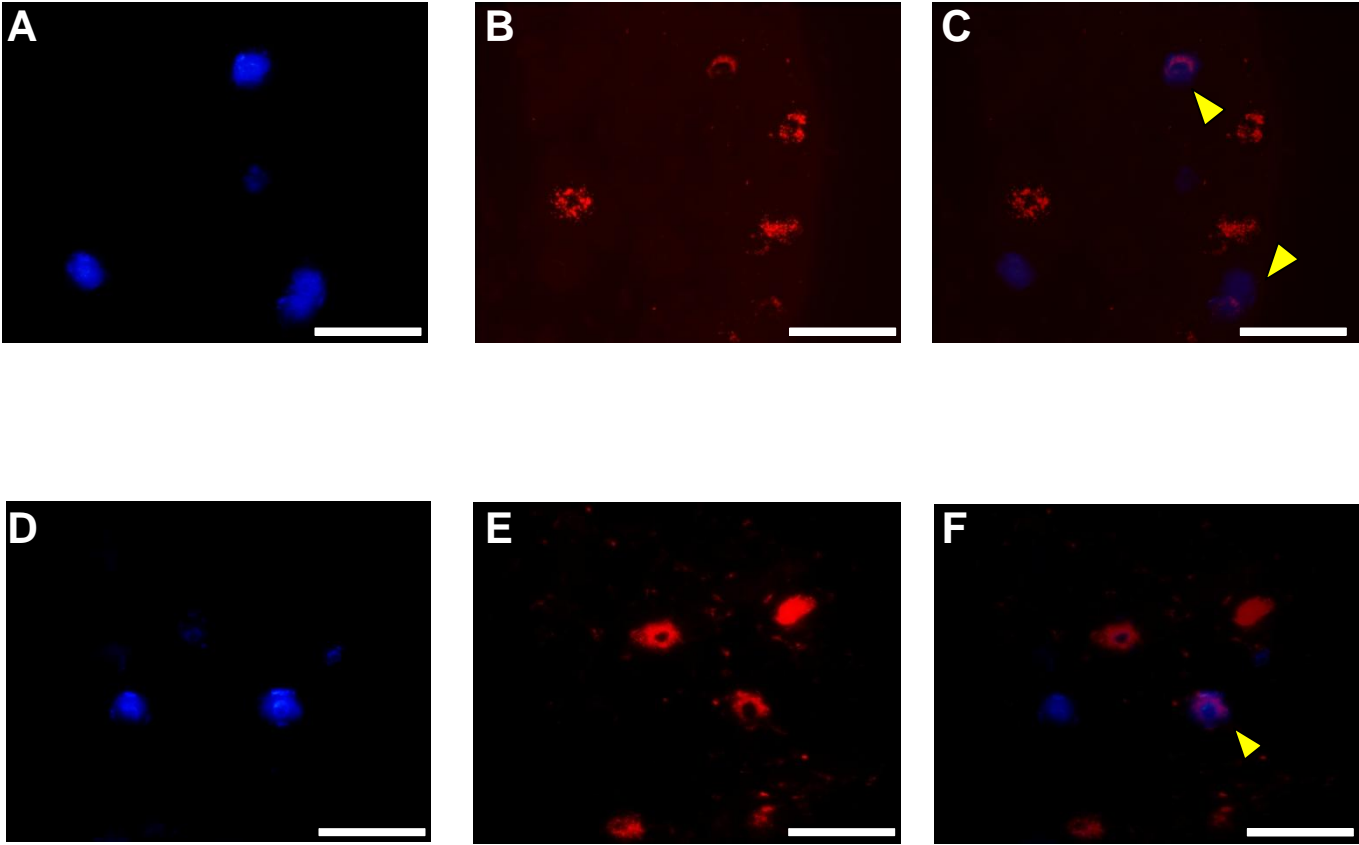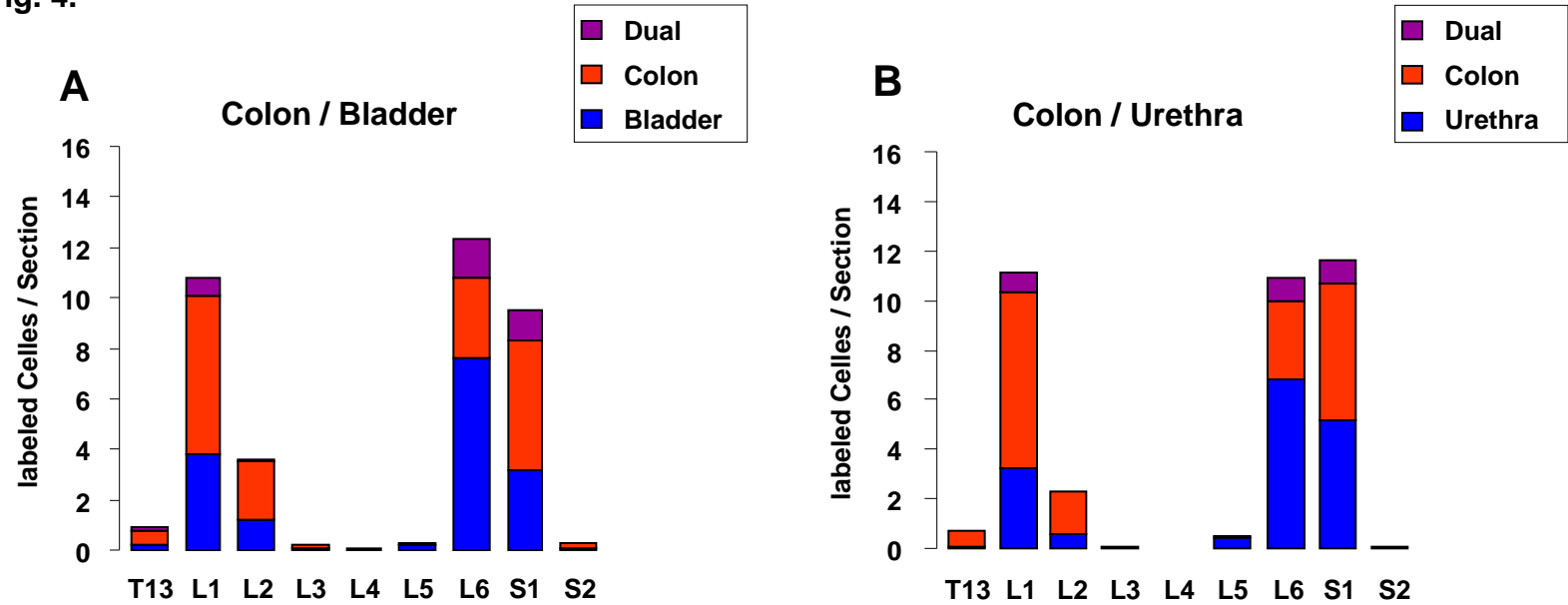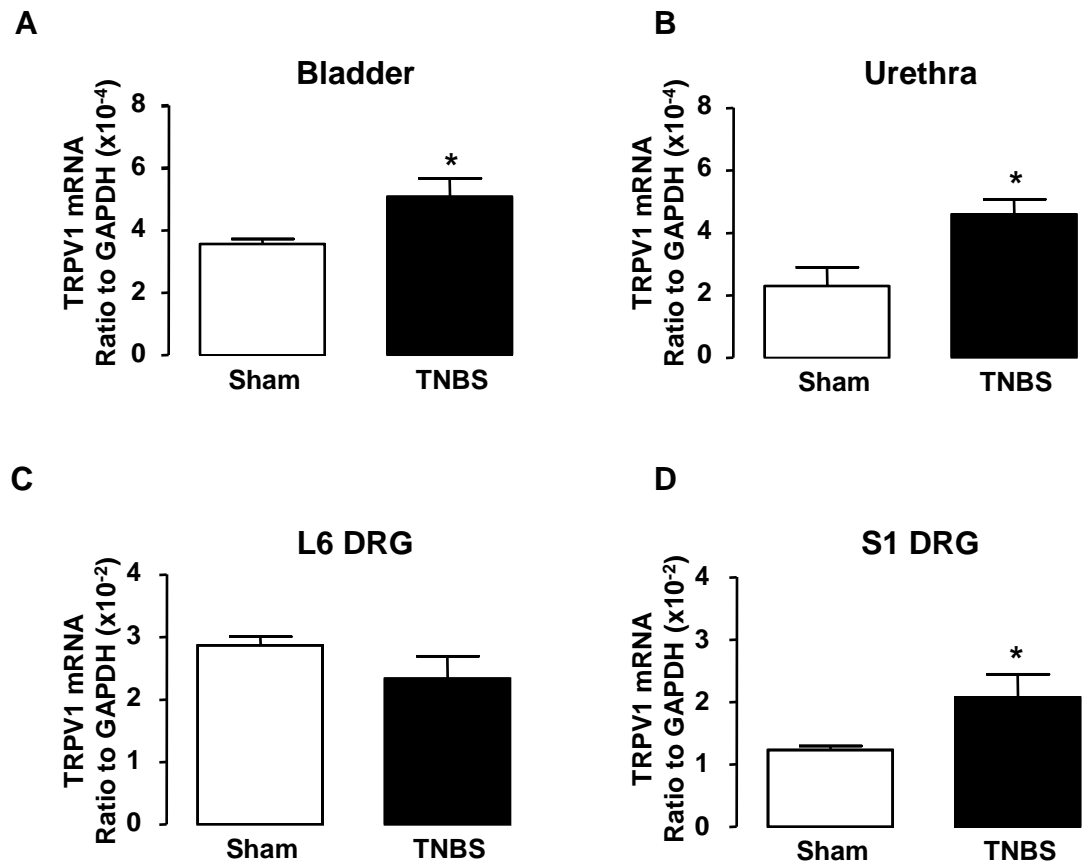ted that dorsal root ganglion (DRG) neurons, innervating the skin and bladder, consist of both IB4-positive and -negative cells (Bennett et al. 1996). More IB4-positive cells are found in skin afferents than bladder afferents, suggesting that the two types of C-fiber neurons may preferentially innervate somatic or visceral tissues (Bennett et al. 1996). However, the proportion of IB4-positive cells in somatic or visceral afferent pathways varies among studies (Ambalavanar et al. 2003; Bennett et al. 1996; Dang et al. 2005; Hwang et al. 2005; Ivanavicius et al. 2004; Lu et al. 2001; Yoshimura et al. 2003).

Voltage-gated K^+ (Kv) currents are major determinants of neuronal excitability. Kv currents in sensory neurons are divided into two major categories: sustained, delayed rectifier-type K^+ (K_{DR}) and transient A-type K^+ (K_A) currents (Gold et al. 1996; Hall et al. 1994; Kostyuk et al. 1981; Yoshimura et al. 1996). K_A currents in DRG cells consist of at least two different components, based on their inactivation kinetics (i.e., fast- and slow-inactivating K_A currents) (Akins and McCleskey 1993; Everill et al. 1998; McFarlane and Cooper 1991). The slow-inactivating K_A current in DRG neurons is partially inhibited by α-dendrotoxin, a Kv1 channel blocker (Yang et al. 2004). Furthermore, chronic bladder inflammation increases excitability of capsaicin-sensitive C-fiber bladder afferent neurons due to reductions in the slow-inactivating K_A current and Kv1.4 channel expression (Hayashi et al. 2009; Yoshimura and de Groat 1999). Thus Kv1.4-containing Kv1 family channels are responsible, at least in part, for the K_A current in visceral afferent neurons. Other potential molecular correlates of the K_A current include Kv4 family channels. Kv4.x subunits constitute a K_A-like, rapidly inactivating channel (Birnbaum et al. 2004). Kv4.3-immunoreactive proteins were found in the somata of a subset of nociceptive DRG neurons (Chien et al. 2007). We also reported recently that Kv.4.1 mRNA is expressed in all sizes of rat DRG neurons, using in situ hybridization techniques (Matsuyoshi et al. 2012). Thus multiple Kv subunits may significantly participate in forming K_A channels, yet molecular correlates of K_A currents in distinct target tissues, cell morphologies, and other properties still remain unclear. AQ: 21

We therefore set out to identify cellular and electrophysiological characteristics of K_A channels in DRG neurons innervating somatic and visceral tissues. Here, we show that IB4 intensely positive neurons innervate the somatic tissue but not the bladder. Moreover, phrixotoxin 2 (PaTx2), a Kv4 channel blocker, exhibits distinct voltage-dependent inhibitions of heterologously expressed Kv4.x currents. With the use of this toxin, we identify further the functional contribution of Kv4.1/4.3 subunits to the K_A channel in IB4 intensely positive C-fiber neurons that contain somatic afferent cells.

Glossary

τ act	Time constants of current activation
τ decay	Time constants of current decay
D	Concentration of toxins (M)
G/G max (control)	Peak K^+ conductance relative to the maximum conductance in control
h	Hill coefficient
I/I max	Normalized peak K^+ current amplitude for inactivation curve analysis
I_c	Mean amplitude of depolarization-induced current in control (in the absence of toxins)
k	Slope factor
K_i	Apparent dissociation constant
L	Fractional current amplitude remaining when the affinity sites are fully occupied
Vh	Voltage at half-maximal conductance

MATERIALS AND METHODS

Animal Preparation and DRG Cell Dispersion

Adult female Sprague-Dawley rats (200–250 g; Hilltop, Pittsburgh, PA) were used. All animal experiments were carried out in accordance with institutional guidelines and were approved by the Institutional Animal Care and Use Committee at the University of Pittsburgh and with the guidelines by the U.S. National Institutes of Health regarding the care and use of animals for experimental procedures. Fast Blue (FB; 2% w/v; EMS-Chemie, Zürich, Switzerland) was injected into the bladder wall to identify afferent neurons innervating the bladder as visceral neurons by retrograde axonal transport of the fluorescent dye, as described previously (Hayashi et al. 2009; Yoshimura and de Groat 1999). Briefly, the bladder was exposed by a midline lower-abdominal incision, and the dye was injected with a 30-gauge needle at four to six sites (20 μl total vol) on the surface of the bladder under isoflurane anesthesia (1–2%). At each injected site, the needle was kept in place for 20–30 s, and any leakage of dye was removed by application of cotton swab. The injection site was then rinsed with saline, and the incision was closed. In a separate group of animals, we injected FB into the dermis surrounding the external urethral orifice at four sites (20 μl total vol) to identify afferent neurons innervating the skin as somatic neurons. After 6–9 days, under isoflurane anesthesia, the L6 and S1 DRG were removed, minced, and incubated for 25 min at 37°C in DMEM (Sigma-Aldrich, St. Louis, MO), containing 0.3 mg/ml trypsin (type III; Sigma-Aldrich), 1 mg/ml collagenase (type I-A; Sigma-Aldrich), and 0.1 mg/ml deoxyribonuclease (type IV; Sigma-Aldrich). Trypsin inhibitor (type II-S; Sigma-Aldrich) was then added to neutralize the enzyme activity. Individual DRG cell bodies were isolated by trituration and plated on poly-D-lysine-coated, 8 mm round coverglasses and incubated at 37°C overnight before patch-clamp experiments. Just before the experiments, single cells were stained by

G. simplicifolia IB4-FITC (3 μg/ml; Axxora, San Diego, CA) for 5 min. The staining condition of IB4-FITC does not influence Kv currents in DRG neurons, including K_A currents (Vydyanathan et al. 2005).

Morphological Characterization in Native DRG Neurons

The coverglass with cultured cells was mounted on a cell chamber for patch-clamp recordings. Microscopic characterization of cells was performed by measuring cell diameter and fluorescence intensities of FB and IB4-FITC by visual inspection (done by one examiner, T.Y.). The degree of IB4 staining in the cell membrane was divided into three grades: *grade 0*, weakly or nonstained (including cytosol-stained cells); *grade 1*, moderately stained on the whole circle of the cell membrane; *grade 2*, intensely stained on the whole circle of the cell membrane.

We also verified this visual grading using the fluorescence intensity of individual cells. Briefly, we quantitatively measured the staining intensity of 92 DRG neurons, randomly photographed during experiments using ImageJ software. The lowest and highest intensities of IB4 staining were rated as 0% and 100%, respectively. With the use of this scale, all visually classified *grades 0*, *1*, and *2* cells fell into the measured fluorescence intensity: <20% of the highest intensity (32 of 92 cells; 34.8%), between 20 and 70% (41 of 92 cells; 44.6%), and >70% (19 of 92 cells; 20.6%), respectively.

Constructions and Transfection

We used Kv4.x channels containing a Myc tag at the N-terminus for this study. Addition of the tag allowed the easy detection of channel protein expression. Myc-tagged Kv4.x, Kv1.4, and Kv2.1 cDNAs were constructed previously in our laboratory (Ren et al. 2003). We targeted the four-amino acid portions in the carboxyl terminal of the S3 region (S3b) to test its roles in voltage dependence of the toxin effects (see Fig. 6). Replacement of the four-amino acid sequences between Kv4.1 and Kv4.2 was done using a two-step, overlapped PCR with primers containing a part of the other channel subunit sequence. Obtained constructs were verified by DNA sequencing.

Chinese hamster ovary (CHO)-K1 cells on an 8-mm coverslip were transfected with expression constructs for Myc-tagged mouse Kv4.1, rat Kv4.2, and rat Kv4.3 at the cDNA ratio 1:4–1:8 using Lipofectamine PLUS (Invitrogen, Life Technologies, Grand Island, NY). We also transfected rat Kv1.4 and rat Kv2.1 on CHO-K1 cells. A small amount of Emerald-C1 (10 ng/dish) was included in transfection for visualization of transfected cells (Takimoto et al. 2002). One or 2 days after transfection, at ~10% confluence, the membrane currents of the cells were recorded using patch-clamp methods.

Whole-Cell, Patch-Clamp Recording Procedure

The setup of the patch-clamp experimental system used was essentially the same as described previously (Hayashi et al. 2009; Yoshimura and de Groat 1999). Briefly, a whole-cell, voltage-clamp recording was performed on dispersed, native DRG neurons or cultured CHO-K1 cells with an Axopatch-700B patch-clamp amplifier, and data were acquired and analyzed by pCLAMP 8 software at a sampling rate of 1,000/s (Axon Instruments, Union City, CA). The filter was set to -3 dB at 2,000 Hz, and the P/N protocol was used to subtract leak currents. Whole-cell input capacitance was neutralized directly from the amplifier. Current traces were low-pass filtered by the digital filter of the data acquisition program (pCLAMP 8). Patch electrodes were fabricated from borosilicate capillary tubing and had resistances of 3–7 MΩ when filled with the internal solution. During recordings, isolated DRG neurons and CHO-K1 cells were superfused with bath solution at a flow rate of 2.0 ml/min in a chamber with a 0.30-ml vol at room temperature (20–22°C). Patch pipettes were filled

with a solution containing the following (in mM): 140 KCl, 1.0 CaCl$_2$, 2.0 MgCl$_2$, 11 EGTA, 2.0 ATP, 0.40 GTP, and 10 HEPES, titrated to pH 7.4 with Tris base.

For patch-clamp experiments in native DRG neurons, we selected two populations of single cells: *1*) FB-positive bladder afferent neurons smaller than 30 μm in diameter and *2*) intensely stained cells with IB4-FITC. Note that there was no overlap in these two populations (see Table 3). We obtained outward K$_{DR}$ currents by depolarizing step pulses from a holding potential of -40 mV for 500 ms, and the outward K$_A$ current was obtained by subtracting K$_{DR}$ from the outward currents activated from the holding potential of -120 mV (I_{-120}), as established previously (Hayashi et al. 2009; Yoshimura and de Groat 1999).

The extracellular solution, which contained the following (in mM): 150 choline-Cl, 5.0 KCl, 0.03 CaCl$_2$, 3.0 MgCl$_2$, 10 D-glucose, and 10 HEPES/Tris-base (pH 7.4), was used to suppress inward Na$^+$ and Ca^{2+} currents. At the end of experiments, all neurons investigated their capsaicin (1 μM) sensitivity by switching the bath solution to the following solution (in mM): 150 NaCl, 5.0 KCl, 2.5 CaCl$_2$, 1.0 MgCl$_2$, 10 D-glucose, and 10 HEPES/Tris-base (pH 7.4). Only the capsaicin-sensitive cells were enrolled into analysis.

In Kv channel-expressed CHO cells, Kv currents were evoked by depolarizing voltage pulses from holding potentials of -100 mV. The extracellular solution included the following (in mM): 150 NaCl, 5.0 KCl, 2.5 CaCl$_2$, 1.0 MgCl$_2$, 10 D-glucose, and 10 HEPES/Tris-base (pH 7.4).

Drugs

All drugs were dissolved directly into the extracellular solution. PaTx2 was applied to the cell by injection into the bath solution. Recombinant PaTx2 was purchased from Alomone Labs (Jerusalem, Israel). The rest of the chemicals were purchased from Sigma-Aldrich.

Equation for Curve Fitting of the Steady-State Activation and Inactivation of K$_A$ and Kv4 Currents

The peak outward current amplitude, evoked by depolarization from the holding potential of -100 mV to various potentials, from -60 to $+50$ mV every 10 mV, was recorded to estimate the peak K$^+$ conductance. The duration from a pulse to the next pulse was 5 s. The steady-state activation curve was plotted as the G/G max (control) vs. the test potential (*V*) concerning the theoretical equilibrium potential of K$^+$ in 20°C. The data were fitted by the modified Boltzmann equation

AQ: 6 $$\text{G/G max (control)} = 1/(1 + \exp[(Vh - V)/k])$$

Steady-state inactivation was determined by depolarizing voltage steps to $+20$ mV, following 1 s conditioning prepulses from -120 to $+20$ mV. The inactivation curve was plotted as the I/I max vs. the potential of the conditioning prepulse (*V*). The data were fitted by the modified Bolzmann equation

$$\text{I/I max} = 1/(1 + \exp[(V - Vh)/k])$$

Equation for Curve Fitting of the Effects of PaTx2 on Expressed Kv4 Currents

The peak inward current amplitude, evoked by depolarization from the holding potential of -100 mV to $+30$ mV for a duration of 300 ms every 15 s (0.067 Hz), was measured to obtain a concentration-response relationship of toxins. The mean amplitude (*I*) of two outward currents, just before application of toxins, was taken as the control level, and the amplitude of current (mean of two stable depolarization-induced currents) during toxin application (4–7 min after application) was estimated. The curve was drawn by fitting the equation using the least squares method

$$I/I_c = L + (1 - L)/[1 + (D/K_i)]^h \qquad \text{AQ: 7}$$

RT-PCR Analysis

Total RNAs were isolated from L6 and S1 DRGs and forebrains, and RT reaction and PCR analyses are performed as described previously (Takimoto et al. 2006). We also performed PCR analyses for Kv4.1 and Kv4.3 mRNA expression in laser-captured DRG neurons. L6 DRGs were removed from rats that had been injected with FB into the bladder wall, 1 wk earlier, and serially sectioned at 8 μm thickness. Every third section was mounted onto polyethylene naphthalate membrane slides (Leica Microsystems, Wetzlar, Germany) to avoid repeated excision of cells. Slides were then stained with IB4-FITC conjugate (50 μg/ml in PBS) for 5 min, rinsed with PBS, and air dried. The slides were observed using a fluorescence microscope with the appropriate filter, and laser capture microdissection (LCM) was performed using the Leica LMD6000 (Leica Microsystems, Buffalo Grove, IL). DRG cells, labeled with FB or stained AQ: 8 with IB4 ($n = 20$ cells each/animal), were laser captured into 0.5 ml Eppendorf tube caps. RNA was isolated from captured individual tissue specimens, followed by DNase treatment. RT and real-time PCR were performed using the TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems, Carlsbad, CA; Tables 1 and T1 2). Gene-specific primers and TaqMan probes were designed with T2 Primer3 Software (Totowa, NJ). Probes contained FAM fluorophore AQ: 9 and TAMRA quencher. The primer and probe combination was AQ: 10 optimized within suitable ranges for efficiency and correlation coefficient using standard curve dilutions and data output on the ABI StepOne Plus thermocycler (Applied Biosystems). Amplification of cDNA was performed under the following conditions: one cycle of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. The reactions were analyzed in triplicate and normalized to GAPDH. Real-time PCR data were analyzed by the difference in crossing point (Cp) method, as R = 2^(Cp sample $-$ Cp AQ:11-12 control) to generate the relative expression ratio (R) of each target AQ: 13 gene relative to GAPDH.

Statistical Analysis

Statistical analyses were performed with nonparametric Mann-Whitney test (see Table 5) and nonparametric Wilcoxon signed-rank

Table 1. *PCR primers for DRG RNAs*

Gene	GenBank #	Sequence	Position	Size, bp
Kv4.1	XM_217601	ACTAGCGGGTCTTCGGAGGA	1,683–1,702	271
		TCACAGGGAAGAGATCTTGA	1,953–1,934	
Kv4.2	NM_031730	TCCGCCACATCCTCAACTTC	828–847	579
		CATCCTCATTGTCTGTCATCAC	1,406–1,385	
Kv4.3	NM_031739	GCAAGACCACGTCACTCATC	1,455–1,474	217 (S)
		AGGTGCGTGGTCTTCTTGCT	1,671–1,652	274 (L)
GAPDH	NM_017008	TATGATGACATCAAGAAGGTGG	787–808	219
		CACCACCCTGTTGCTGTA	1,005–988	

DRG, dorsal root ganglion; Kv, voltage-gated K$^+$; S, short splicing isoform of Kv4.3; L, long splicing isoform of Kv4.3.

Table 2. *Real-time PCR primers and probes for laser-captured cells*

Gene	GenBank #		Sequence	Position
Kv4.1	NM_001105748	5′ Primer	GCACCAGCAAGACCAACTTC	1,049–1,068
		3′ Primer	GTACAGGCAAGGCAATGACC	1,213–1,194
		Probe	TGGTGCCTAGCACCATCGCA	1,133–1,152
Kv4.3	NM_031739	5′ Primer	CAAGACCACGTCACTCATCG	1,490–1,509
		3′ Primer	AGAGAAGGGCTTCTGGTGGA	1,642–1,623
		Probe	TGAGCAGAACTGCATGGAGAGCTCA	1,583–1,607
GAPDH	NM_017008	5′ Primer	AGACAGCCGCATCTTCTTGT	29–48
		3′ Primer	GATACGGCCAAATCCGTTC	112–94
		Probe	GCAGTGCCAGCCTCGTCTCA	49–68

AQ: 14

test (see Figs. 3 and 7, and see Table 6). Changes were considered significant at $*P < 0.05$, and $**P < 0.01$. Data are expressed as mean with the SE, except those (see Table 5) that were expressed as mean with SD.

RESULTS

Characterization of Bladder and Cutaneous DRG Neurons

F1

T3

We first examined IB4-FITC binding to acutely dissociated, live L6-S1 DRG neurons from animals that had been injected with FB at the bladder walls. Dissociated, single DRG neurons (530 cells) were observed as round- to oval-shaped cells, in which 11% (59 cells) were FB-positive bladder afferent neurons (Fig. 1A). Staining with IB4-FITC provided various fluorescence intensities. We classified cells based on the IB4-staining level into three categories: cells showed no or negligible fluorescent staining (IB4:0), whereas others exhibited moderate to intense signals (moderate, IB4:1; intense, IB4:2; Fig. 1 and Table 3). The analysis of 530 cells from three rats in this fashion indicated that >⅓ of cells were moderately stained with IB4, whereas ~20% of cells were intensely stained (Table 3). IB4-stained cells were distributed in various

sizes of DRG neurons (Fig. 1B). We also verified visual evaluation of IB4-FITC signal intensity by measuring the fluorescence intensity of 92 DRG neurons, randomly photographed during experiments. The fluorescence intensity of *grades 0, 1*, and *2* cells, judged by visual evaluation, was <20% (32 of 92 cells; 34.8%), between 20 and 70% (41 of 92 cells; 44.6%), and >70% (19 of 92 cells; 20.6%) of the highest value among DRG neurons, respectively, demonstrating that our visual evaluation method is appropriate to classify DRG neurons based on IB4-staining intensity. FB-positive bladder afferent neurons showed only negative to weak (IB4:0, 35/59 cells = 59%) or moderate (IB4:1, 24/59 cells = 41%) IB4-FITC staining and were never intensely stained with IB4-FITC (Fig. 1A).

L6-S1 DRG also contains somatic afferent neurons innervating the external skin area surrounding the urethra through the pudendal nerve. Therefore, we analyzed a total of 260 cells from three rats that had been injected with FB at the external urethral dermis (Fig. 2 and Table 4). Among those, 34 cells (13%) were FB-positive cutaneous afferent neurons. As expected, three IB4-staining categories represented similar proportions to the above experiments. Likewise, no apparent

F2,T4
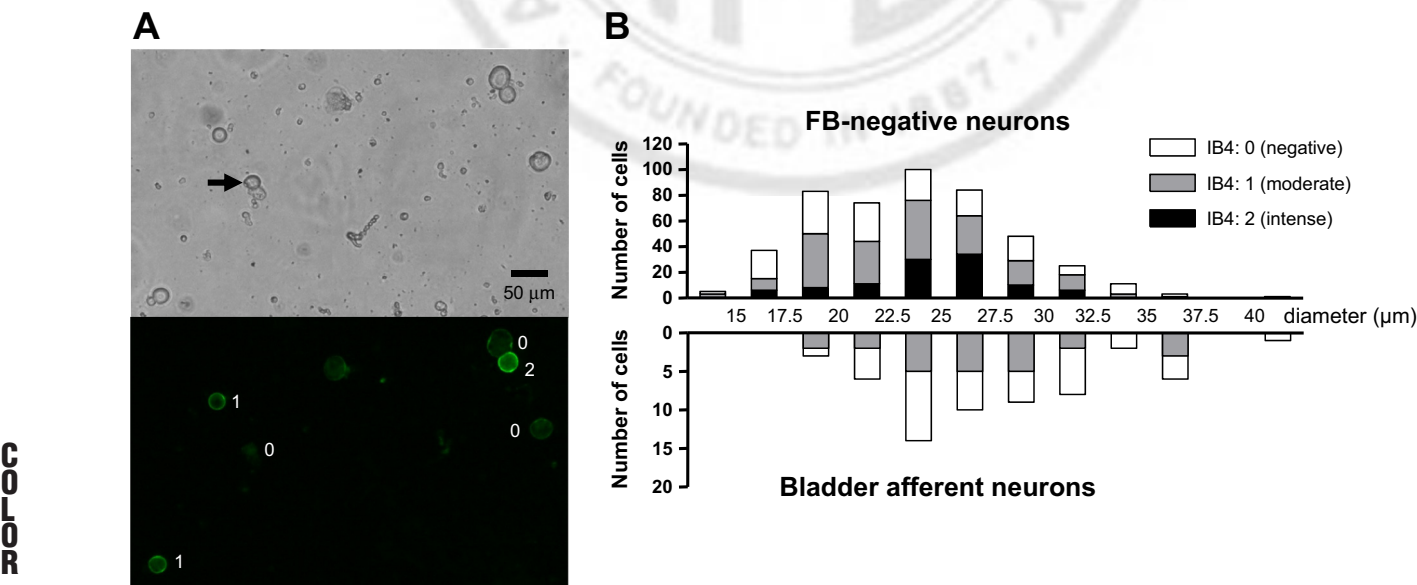
Fig. 1. Morphological characterization of dissociated L6-S1 dorsal root ganglion (DRG) neurons from rats injected with Fast Blue (FB) into the bladder wall. *A*: single DRG neurons in the same field, observed by light-field and fluorescent microscope, are shown in the *top* and *bottom*, respectively. *Top*: an arrow indicates a FB-positive bladder afferent neuron identified under the fluorescent light. *Bottom*: single DRG neurons with various green-staining intensities of isolectin B4 (IB4)-FITC. The numbers (0–2) beside the cells indicate the intensity of staining: 0, no or weak (negative) staining; 1, moderate staining; 2, intense staining. *B*: cell-size distributions of FB-positive and -negative DRG cells with different IB4 staining intensities (*grades 0–2*). Note that there is no *grade 2*-stained cells in FB-positive bladder afferent neurons.

Table 3.   *IB4 staining intensity of bladder afferent neurons*

	IB4:0	IB4:1	IB4:2
FB-negative neurons	168 (32%)	198 (37%)	105 (20%)
Bladder afferent neurons	35 (6.6%)	24 (4.5%)	0 (0%)
Total neurons	203 (38%)	222 (42%)	105 (20%)

Total 530 cells. Fast Blue (FB) was injected into the bladder wall at 4–6 sites (20 μl total vol) in each animal. IB4, isolectin B4.

Table 4.   *IB4 staining intensity of skin afferent neurons*

	IB4:0	IB4:1	IB4:2
FB-negative neurons	90 (35%)	96 (37%)	40 (15%)
Skin afferent neurons	11 (4.2%)	14 (5.4%)	9 (3.5%)
Total neurons	101 (39%)	110 (42%)	49 (19%)

Total 260 cells. FB was injected into the skin surrounding the external urethral orifice at 4 sites (20 μl total vol) in each animal.

correlation between IB4-staining intensity and cell size was observed. However, FB-positive cutaneous afferent neurons included many IB4-stained cells, and >¼ of FB-positive cells were intensely stained with IB4-FITC (IB4:2, nine/34 cells). Thus intense IB4-positive cells constitute a subpopulation of somatic afferent neurons innervating the urethral external skin, whereas visceral afferent neurons innervating the bladder consist of IB4-negative to moderately stained cells. Hence, afferent neurons with distinct IB4-staining intensities may preferentially innervate either cutaneous or visceral tissues.

Distinct Effects of PaTx2 on K_A Currents in Bladder Afferent and IB4-Positive Neurons

K_A currents play important roles in controlling activity of excitable cells, including sensory neurons. We wished to identify functional differences in K_A currents between cutaneous and visceral afferent neurons. We chose two populations of L6-S1 DRG neurons, <30 μm in diameter, from rats injected with FB in the bladder: *1*) IB4 intensely stained cells and *2*) FB-positive cells. The former represents a subpopulation of somatic afferent neurons, whereas the latter corresponds to a fraction of bladder afferent neurons. There was no overlap in these two neuron types. All of the tested cells in the two groups showed the response to capsaicin (1 μM) to induce inward current, indicating that they were capsaicin-sensitive C-fiber afferent cells. The peak current density of the capsaicin-induced inward current was 80 $\pm$ 4.7 pA/pF ($n = 24$) in IB4 intensely stained neurons and 89 $\pm$ 12 pA/pF in bladder afferent neurons ($n = 11$).

Kv currents were recorded using two holding potentials to obtain K_{DR} and K_A current components. K_{DR} currents were evoked from a holding potential of -40 mV, whereas K_A currents were obtained by subtracting K_{DR} currents from I_{-120}, as we reported previously (Hayashi et al. 2009; Yoshimura and de Groat 1999). Peak amplitudes of K_A and K_{DR} were larger in IB4 intensely stained cells than those in bladder afferent cells ($P < 0.01$, Mann-Whitney test; Table 5). The *Vh* in **T5** steady-state activation and inactivation was lower in IB4 intensely stained cells than those in bladder afferent neurons ($P < 0.01$ for steady-state activation, and $P < 0.01$ for steady-state inactivation, Mann-Whitney test; Table 5). These differences indicate that the bladder afferent cells possess a typical slowly inactivating K_A current, as we have reported previously (Yoshimura and de Groat 1999; Yoshimura et al. 1996), whereas IB4 intensely positive cells display K_A currents with faster kinetics.

Recent studies indicate that Kv4.x subunits are significantly expressed in DRG neurons (Matsuyoshi et al. 2012; Phuket and Covarrubias 2009). To identify the functional contribution of Kv4 channels to K_A currents in DRG neurons, we tested the effect of a Kv4 blocker, PaTx2, on the currents. Application of PaTx2 at 1 μM significantly inhibited the K_A current in IB4 intensely positive cells ($P < 0.01$ for PaTx2, 500 nM; $P < 0.01$ for PaTx2, 1,000 nM; Wilcoxon signed-rank test) without significant influence on the K_{DR} current (96 $\pm$ 0.85% of the predrug value for PaTx2, 1,000 nM, $n = 13$; Fig. 3, *A*, *B*, and **F3** *D*). In contrast, PaTx2 produced no effect on the K_A currents in **AQ: 15** FB-positive bladder afferent neurons (Fig. 3, *C* and *D*). These results indicate that Kv4 family channels contribute to the K_A current in IB4 intensely stained afferent neurons that represent a subpopulation of somatic afferent cells. In contrast, these
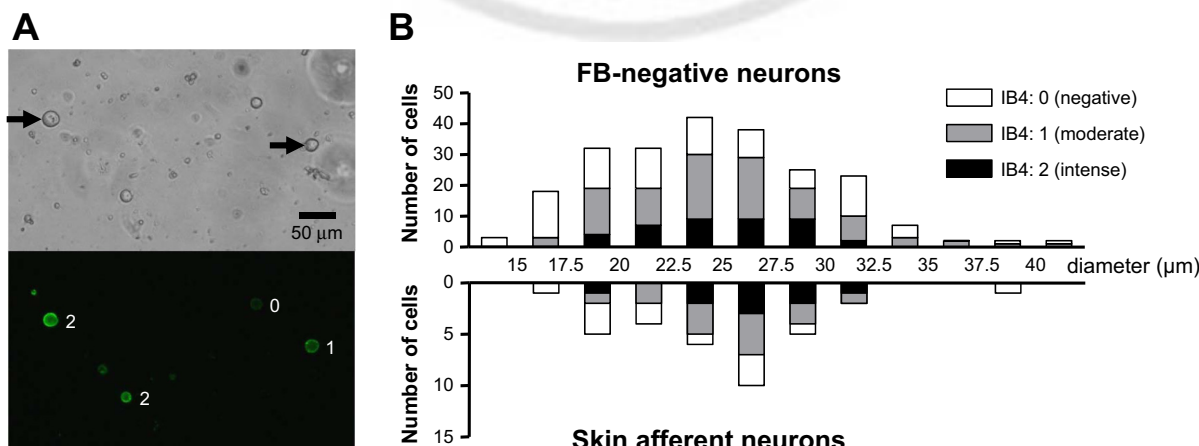
Fig. 2. Morphological characterization of dissociated L6-S1 DRG neurons from rats injected with FB into the skin around the urethral orifice. *A*: single DRG neurons in the same field observed by light-field and fluorescent microscope are shown in the *top* and *bottom*, respectively. *Top*: arrows indicate FB-positive cutaneous afferent neurons identified under the fluorescent light. *Bottom*: single DRG neurons with various green-staining intensities of IB4-FITC. The numbers (0–2) beside the cells indicate the intensity of staining, as described in Fig. 1. *B*: cell-size distributions of FB-positive and -negative DRG cells with different IB4-staining intensities (*grades 0–2*). Note that *grade 2*-stained cells are observed in FB-positive cutaneous afferent neurons.

Table 5. *Electrophysiological properties of IB4 intensely positive and bladder afferent neurons*

	IB4 Intensely Positive Cells	Bladder Afferent Cells
Number of cells	21	9
Diameter, μm	27 ± 2.1	$24 \pm 2.2^*$
Input capacitance, pF	35 ± 3.7	$28 \pm 3.3^*$
I_A peak at 0 mV, nA	2.5 ± 0.63	$0.73 \pm 0.14^*$
I_{DR} peak at 0 mV, nA	1.3 ± 0.39	$0.75 \pm 0.22^*$
Steady-state activation Vh, mV	-58 ± 4.4	$-39 \pm 5.0^*$
Steady-state inactivation Vh, mV	-89 ± 4.3	$-75 \pm 4.3^*$
τ act at 0 mV, ms	1.9 ± 0.20	$3.5 \pm 0.72^*$
τ decay at 0 mV, ms	160 ± 19	$210 \pm 17^*$

Values are means $\pm$ SD. I_A, A-type transient K^+ current; I_{DR}, delayed rectifier-type K^+ current. *$P < 0.01$ vs. IB4 intensely positive cells, Mann-Whitney test.

channels do not contribute significantly to the K_A current in bladder afferent neurons.

PaTx2 is closely related to the gating-modifying toxins that act on Kv2 and/or Kv4 channels, such as hanatoxins and heteropoda toxins (Diochot et al. 1999; Escoubas et al. 2002; Sanguinetti et al. 1997; Swartz and MacKinnon 1995). These toxins usually exhibit voltage-dependent inhibition of target channel currents. We found that PaTx2 similarly inhibited the K_A current in IB4 intensely positive DRG neurons at all

depolarizing pulses (Fig. 4*A*). No apparent voltage shift was **F4** seen in the normalized steady-state activation curves before and after application of PaTx2 (Fig. 4*B*). The toxin produced no significant changes in time constants for activation and inactivation, although it tended to slow both processes at all voltages (Fig. 4, *C* and *D*). Thus PaTx2 voltage independently blocks the K_A current in IB4 intensely stained cells without apparent effects on activation or inactivation.

Distinct Effects of PaTx2 on Heterologously Expressed Kv4 Channels

The lack of voltage dependence of the toxin action on the native K_A current stimulated us to test whether the toxin might similarly inhibit heterologously expressed Kv4 currents in a voltage-independent fashion. We expressed N-terminally Myc-tagged rat Kv4.1, Kv4.2, or Kv4.3 channels in CHO cells. Transient outward K^+ currents were evoked by depolarizing voltage pulses in channel cDNA-transfected cells (Fig. 5, **F5** *A–C*). The inactivation time courses were slightly slower than those obtained with the corresponding channels without a tag (time constants of inactivation at 30 mV were 56.3 ± 6.8 ms for wild-type Kv4.3 and 104.7 ± 22.3 ms for Myc-tagged Kv4.3; unpublished observation). The toxin at 500 nM significantly reduced the peak amplitude of all Kv4.x currents ($P < 0.01$ for all Kv4.x currents evoked with depolarization to 0 mV
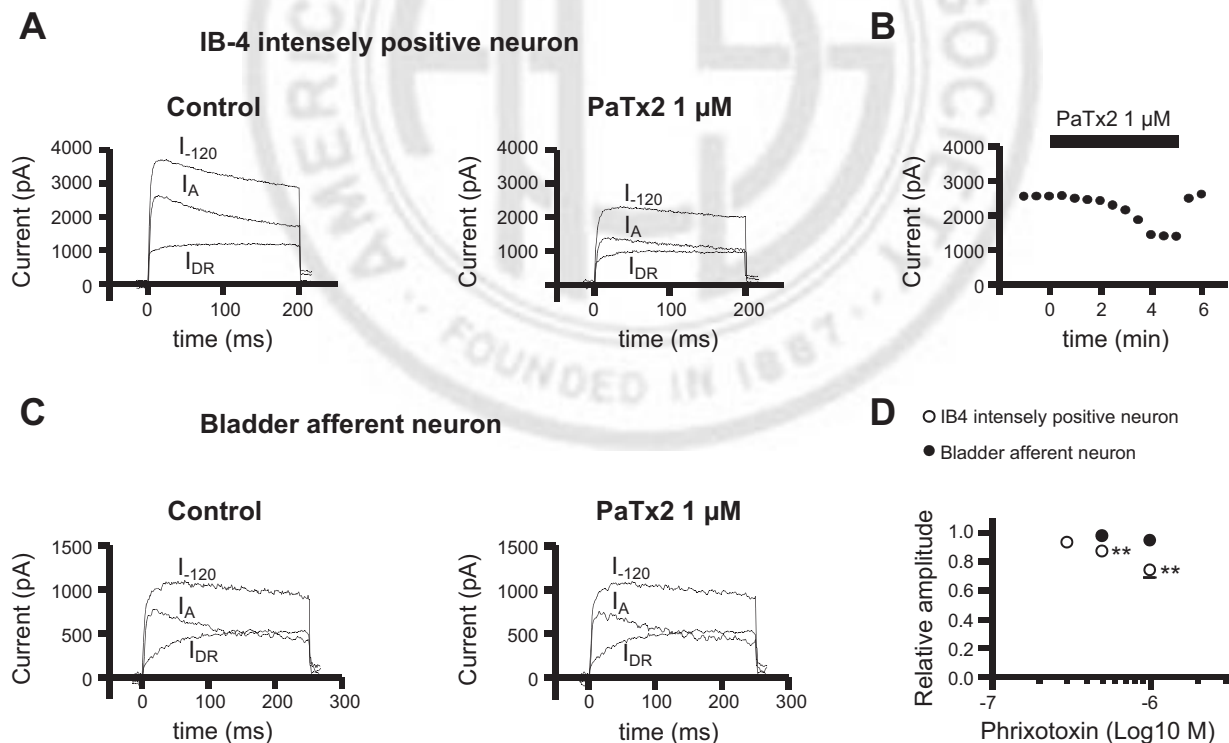
Fig. 3. Effects of phrixotoxin 2 (PaTx2) on A-type transient K^+ (K_A) currents in IB4 intensely stained and bladder afferent neurons. K_A current (I_A) trace was obtained by subtracting the delayed rectifier-type K^+ (K_{DR}) current (I_{DR}) from the outward currents activated from the holding potential of -120 mV (I_{-120}). I_{DR} and I_{-120} were evoked by a depolarizing voltage step to 0 mV from the holding potential of -40 mV and -120 mV, respectively. *A* and *C*: representative I_{-120}, I_{DR}, and I_A before (Control) and after application of 1 μM PaTx2 in an IB4 intensely positive neuron (*A*) and a bladder afferent neuron (*C*). *B*: the time course of PaTx2 effects on peak I_A amplitudes in an IB4 intensely positive neuron. *D*: the concentration-response relationship of PaTx2. Relative peak I_A amplitudes were determined using the level before application of PaTx2 (control) as 1 from the same cell. In IB4 intensely positive cells, PaTx2 decreased the K_A current in a concentration-dependent manner: 300 nM, 0.93 ± 0.024; 500 nM, 0.87 ± 0.019; 1,000 nM, 0.74 ± 0.040 ($n = 4 \sim 18$). Peak I_A amplitudes at 500 and 1,000 nM PaTx2 were significantly different from the control level in IB4 intensely positive cells ($P < 0.01$ for PaTx2, 500 nM; $P < 0.01$ for PaTx2, 1,000 nM, Wilcoxon signed-rank test). In contrast, PaTx2 exhibited no significant changes in peak I_A amplitudes in bladder afferent neurons: 500 nM, 0.98 ± 0.0050; 1000 nM, 0.94 ± 0.023 ($n = 6 \sim 11$). The data are expressed as means $\pm$ SE.
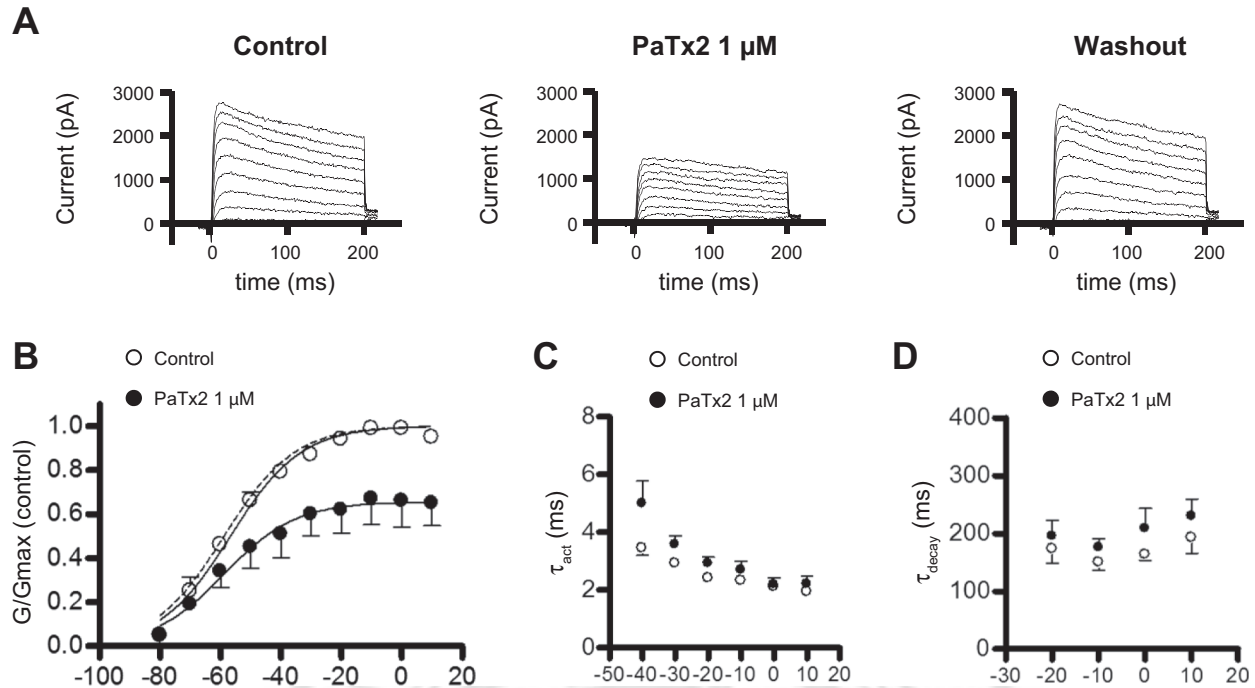
Fig. 4. Effects of PaTx2 on steady-state kinetic parameters of I_A in IB4 intensely stained neurons. I_A current trace was obtained by subtracting K_{DR} from I_{-120}. Membrane depolarization was evoked by test pulses from -80 to $+10$ mV with a 10-mV interval from the holding potential of -40 mV (K_{DR}) and -120 mV (I_{-120}). *A*: representative I_A current traces show inhibition by PaTx2 and its recovery. I_A in IB4 intensely positive DRG cells was suppressed by application of 1 μM PaTx2 and was almost totally recovered after washing PaTx2 by perfusion. *B*: relative conductance levels (G/G max control) are plotted against the depolarizing test potential (*V*). Open and filled circles indicate the values before and after application of PaTx2, respectively. Solid lines were obtained with the relative conductance levels in the absence and presence of 1 μM PaTx2. A broken line was generated with the normalized PaTx2 data using the maximum conductance value. *C*: τ act are plotted against *V*. *D*: τ decay are plotted against *V*. Note that time-constant (τ) values for activation and inactivation were obtained using a single exponential equation. The data are expressed as means $\pm$ SE ($n = 5$).

and $+30$ mV, Wilcoxon signed-rank test; Fig. 5, *A–C*). The estimated K_i values were 230 nM, 120 nM, and 110 nM for Kv4.1, Kv4.2, and Kv4.3 currents evoked with depolarization to $+30$ mV, respectively (Table 6). No detectable effects were seen with Kv1.4 or Kv2.1 currents at 500 nM (data not shown). Hence, PaTx2 is specific for Kv4 family channels in this concentration range.

Importantly, we found that PaTx2 produced distinct inhibitory actions on Kv4 currents. The toxin similarly reduced Kv4.1 current elicited by pulse voltages at 0 and $+30$ mV, whereas it caused more pronounced inhibition of Kv4.2 and Kv4.3 currents at the lower pulse voltage (Fig. 5*D*). The toxin did not apparently change the voltage dependence of steady-state activation (Fig. 5*A*) or inactivation (Fig. 5*E*) for the Kv4.1 current. In addition, no significant changes in the time constants for activation or inactivation were observed at any pulse voltages for the current (Fig. 5*A*). In contrast, the toxin caused clear changes in these parameters for Kv4.2 and Kv4.3 currents. The steady-state activation (Fig. 5, *B* and *C*) and inactivation (Figs. 5*E*) curves were shifted to more positive voltages with significant changes in the time constants for activation and inactivation (Fig. 5, *B* and *C*). These results indicate that the toxin voltage independently and dependently inhibited Kv4.1 and Kv4.2/Kv4.3 currents, respectively. It should be noted, however, that Kv4.3 current exhibited a complex behavior upon the toxin treatment and might involve, in part, voltage-independent inhibition by the toxin. For instance, the current showed a less obvious, positive shift in the steady-state inactivation (Fig. 5*E*).

The observed different inhibitory modes of Kv4 channels might arise from sequence differences in their interaction site with PaTx2. Specifically, the latter part of the third transmembrane S3 is a major interaction site with gating modifier toxins. Sequence alignment of Kv4 polypeptides revealed divergence of four amino acids in this portion (Fig. 6*A*). Therefore, we set out to test if swapping this portion between Kv4 proteins might transfer voltage dependence of the toxin action. Since Kv4.1 and Kv4.2 channels show apparent differences in the toxin inhibition, we generated the two-channel proteins with the four-amino acid sequence of the other at the corresponding site (Kv4.1-VMTD and Kv4.2-FVPK). Kv4.1-VMTD exhibited most of the characteristics of the wild-type Kv4.2 channel in the toxin inhibition. The toxin produced larger inhibition at lower pulse voltage (Fig. 6*D*) and shifted steady-state activation and inactivation curves to the right (Fig. 6, *B* and *E*). Time constants for inactivation were also reduced at lower voltages. Similarly, Kv4.2-FVPK showed voltage-independent inhibition by the toxin. The toxin similarly decreased Kv4.2-FVPK current at different pulse voltages (Fig. 6*D*) without an apparent shift in the steady-state inactivation curve and time constants (Fig. 6, *E* and *B*). These results indicate that the four-amino acid portion at the end of S3 determines voltage dependence of the inhibition by PaTx2.

Expression of Kv4 mRNAs in DRG Neurons

We performed RT-PCRs to detect the expression of Kv4 mRNAs. Abundant expression of Kv4.1 and the long isoform of Kv4.3 mRNAs were seen in L6-S1 DRGs (Fig. 7*A*). In
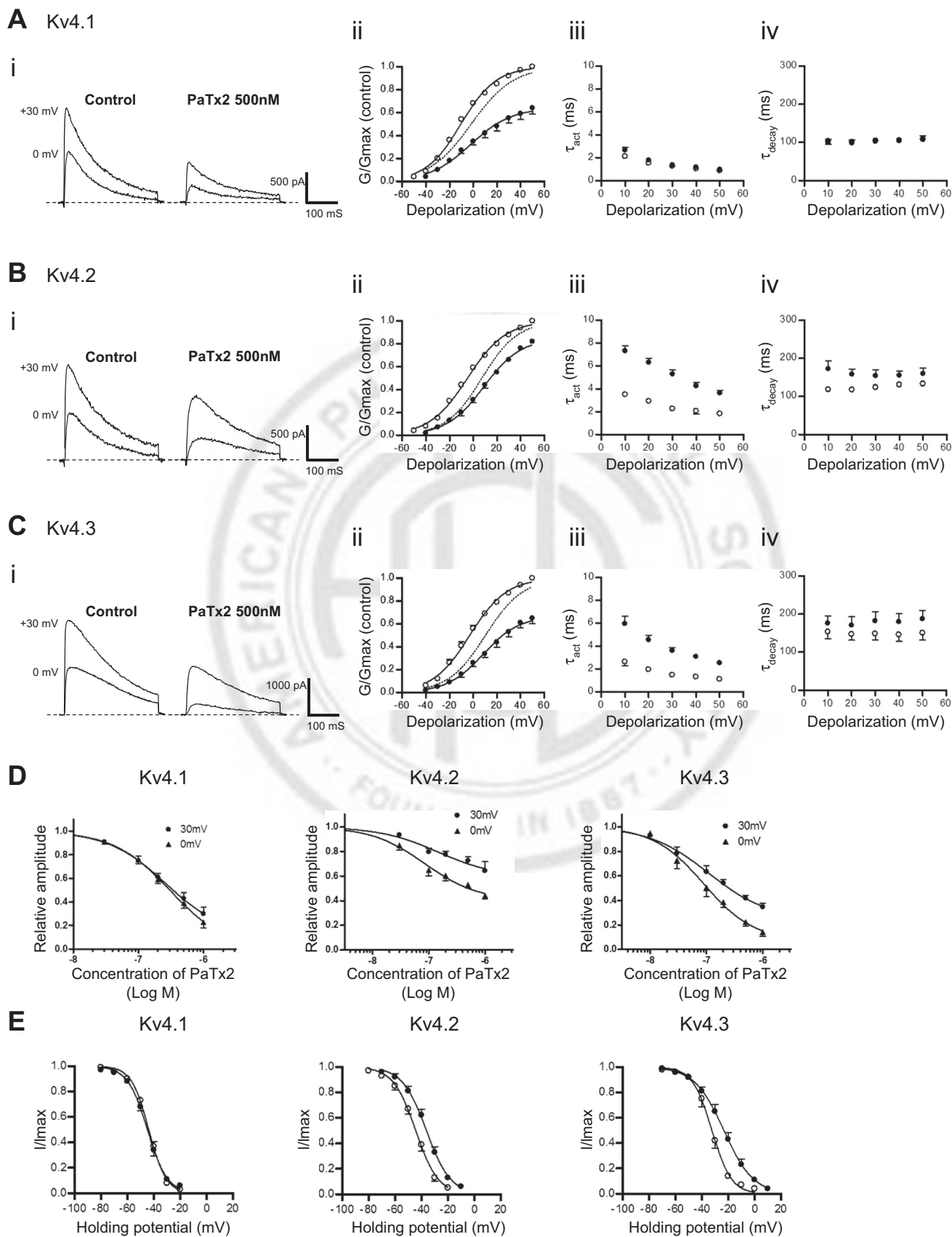
Table 6. *Effects of PaTx2 on Kv4 channels expressed in CHO cells*

		Kv4.1	Kv4.2	Kv4.3	Kv4.1 VMTD	Kv4.2 FVPK
K_i value, nM	0 mV	250	80	89	55	650
	30 mV	230	120	110	36	810
Steady-state activation V_h, mV	Control	−11 ± 1.7	−4.8 ± 1.4	−3.1 ± 1.6	−2.4 ± 2.2	−2.9 ± 1.9
	PaTX2, 200 nM	−3.8 ± 2.0*	7.6 ± 1.7*	12 ± 3.3*	12 ± 2.9†	3.4 ± 2.8*‡
Steady-state activation slope factor	Control	15 ± 0.55	16 ± 0.53	15 ± 0.34	15 ± 0.58	16 ± 0.40
	PaTX2, 200 nM	18 ± 0.37*	15 ± 0.55	17 ± 0.81	14 ± 0.73	17 ± 0.50‡
Steady-state inactivation V_h, mV	Control	−44 ± 0.90	−44 ± 1.8	−32 ± 1.7	−36 ± 1.7	−42 ± 1.2
	PaTX2, 500 nM	−45 ± 1.3	−36 ± 1.8*	−23 ± 2.3†	−18 ± 1.9*	−44 ± 0.88
Steady-state inactivation slope factor	Control	6.4 ± 0.26	8.3 ± 0.46	6.7 ± 0.37	5.5 ± 0.14	8.4 ± 0.27
	PaTX2, 500 nM	7.5 ± 0.47	8.8 ± 0.71	10 ± 0.56†	8.3 ± 0.41*	9.2 ± 0.55

CHO, Chinese hamster ovary. Values are means ± SE ($n = 5$–11). *$P < 0.05$, and †$P < 0.01$ vs. respective control values, Wilcoxon signed-rank test; ‡values obtained after application of phrixotoxin 2 (PaTX2), 500 nM.

contrast, a very low level of the Kv4.2 message was detected. These findings indicate that DRG neurons are largely devoid of Kv4.2.

We then compared the expression level of Kv4.1 and Kv4.3 mRNAs between bladder afferent neurons and IB4-stained DRG neurons using LCM methods (Fig. 7B). Although the difference in IB4-staining intensity (e.g., intense vs. moderate staining) is less obvious in DRG sections (Fig. 7B) compared with the cell culture system (Figs. 1 and 2), we laser captured DRG cells, which were not labeled by FB injected into the bladder wall, with relatively strong IB4 staining. Real-time PCR analysis detected higher expression of Kv4.1 and Kv4.3 mRNAs in laser-captured, IB4-stained neurons than in FB-labeled bladder afferent neurons ($n = 6$ rats; $P < 0.05$ for both Kv4.1 and Kv4.3, Wilcoxon signed-rank test; Fig. 7, D and E).

Taken together, the findings suggest that selective expression of the pore-forming Kv4.1 and possibly Kv4.3 in IB4 intensely stained afferent neurons contributes to the observed voltage-independent inhibition of the K_A current.

DISCUSSION

IB4 Staining of Bladder and Cutaneous Afferent Neurons in Rats

Several groups have reported various percentages of IB4-positive cells in adult rat bladder afferents (L6-S1): 14% (adult female) (Yoshimura et al. 2003), 29% (adult male) (Bennett et al. 1996), 48% (adult male) (Hwang et al. 2005), and 61% (adult male) (Dang et al. 2005). Dang et al. (2005) reported the highest rate of IB4 staining in bladder afferents, possibly due to the high-intensity staining by IB4-Alexa Fluor 488 used in their study, whereas IB4-FITC was used in other studies, including our current study. The former two groups also reported that the percentage of IB4-positive cells is higher in somatic afferent neurons than in bladder afferents: 27% (distal urethra, L6-S1 DRG) (Yoshimura et al. 2003) and 43% (medial ankle, L3 DRG) (Bennett et al. 1996). Other studies have also found high percentages of IB4-positive cells in cutaneous somatic afferent neurons from lumber DRG of adult rats: 48% (knee) (Ivanavicius et al. 2004), 44% (vibrissal pad area) (Ambalavanar et al. 2003) using IB4-FITC, and 70% (foot-pads) (Lu et al. 2001) using IB4-Cy3.

In our current experiments with single, live DRG neurons, membrane staining of IB4-FITC was compared very clearly with an image of fixed and sliced sections. This condition with live cells allowed us to classify the level of IB4 staining. Instead of defining individual neurons as positive or negative, we therefore divided cells into three categories: *grades 0* (no or weak staining), *1* (moderate staining), and *2* (intense staining). According to this classification, we found that bladder afferent neurons lack the IB4 intensely stained (*grade 2*) cells. On the contrary, skin afferent neurons contained significant IB4 intensely stained (*grade 2*) cells (24%). These results strongly suggest that IB4 intensely stained (*grade 2*) cells are not visceral afferents but represent a subpopulation of somatic afferent neurons. On the other hand, IB4 moderately stained (*grade 1*) cells were included in both bladder and skin afferent populations. Taken together, various percentages of IB4-positive cells in visceral and somatic DRG neurons in the previous studies might be attributable to different criteria used to identify IB4-positive cells. In particular, the previous studies might include or exclude in the positive category IB4 moderately stained cells, which were categorized as *grade 1* in the current study.

Electrical and Pharmacological Properties of K_A Currents in Bladder Afferent and IB4 Intensely Positive Neurons

The IB4 staining results indicate that DRG cells, intensely stained with IB4-FITC, are a subpopulation of somatic afferent neurons but not visceral afferent neurons innervating the bladder. Thus the combination of FB injection into the bladder wall with IB4 staining of live, dissociated L6-S1 DRG cells allowed us to examine the differences in potassium currents in the same preparation. In this preparation, FB-positive cells were bladder-innervating visceral afferent neurons, whereas IB4 in-

Fig. 5. Effects of PaTx2 on expressed voltage-gated K^+ (Kv)4 currents. Kv4.1, Kv4.2, and Kv4.3 currents were recorded in Chinese hamster ovary-K1 cells transfected with Myc-tagged rat Kv4.x expression vectors in a whole-cell configuration. K^+ currents were elicited by 300 ms test pulses from a holding potential of −100 mV, before and after application of 200 nM PaTx2, except that the data with 500 nM toxin are shown in *Ai, Bi, Ci,* and *E*. Open and closed circles represent before and after application of 200 nM PaTx2 ($n = 5$–8). *A–C, i*: representative Kv4 current traces; *ii*: steady-state activation curves; *iii*: τ act; and *iv*: τ decay. Broken lines (in *ii*) were generated with the PaTx2 data normalized to the maximum conduction value. τ Values for activation and inactivation were obtained using a single exponential equation. *D*: PaTx2 dose-dependent inhibition of Kv4 currents at 0 mV and 30 mV. *E*: steady-state inactivation.

Fig. 7. Expression of Kv4 mRNAs in DRG. *A*: RT-PCR data with tissue samples. PCR was performed with primers for Kv4.x designed to detect splicing variants and the housekeeping gene, GAPDH. The 2 bands with distinct sizes in Kv4.3 correspond to the short and long splicing isoforms (Kv4.3S and Kv4.3L) (Takimoto et al. 1997). The *left* lane indicates molecular size markers (φX174 DNA digested with *Hae*III). Note that DRGs contain abundant Kv4.1 and Kv4.3L but not Kv4.2. *B*: photomicrographs of the same L6 DRG section taken during laser-captured microdissection (LCM) of FB-labeled bladder afferent neuron before (*left* and *middle*) and after (*right*) LCM. An arrow indicates a laser-captured bladder afferent neuron labeled with FB injected into the bladder wall (*left*). A green-line circle represents the area captured by this procedure. *C*: photomicrographs of the same L6 DRG section taken during LCM of IB4-stained DRG neurons before (*left* and *middle*) and after (*right*) LCM. Arrows point to DRG neurons positively stained with IB4 (*left*). A red-line circle indicates the area that was laser captured (*middle*). *D*: Kv4.1 mRNA levels in laser-captured, FB-labeled bladder afferent neurons and IB4-stained DRG neurons. *E*: Kv4.3 mRNA levels in laser-captured, FB-labeled bladder afferent neurons and IB4-stained DRG neurons. *D* and *E*: data are expressed as relative values normalized with GAPDH mRNA as a control. Note that Kv4.1 and Kv4.3 mRNA levels are significantly higher in IB4-stained cells than those in FB-labeled bladder afferent neurons.

AQ:19-20

tensely stained neurons should represent a fraction of IB4-positive somatic afferent neurons. We found the following using these techniques: that *1*) IB4 intensely stained DRG neurons exhibit a larger amplitude of K_A currents than FB-labeled bladder afferent neurons, *2*) activation and inactivation of K_A currents in IB4 intensely stained DRG neurons are faster with more hyperpolarized, half-maximum potentials than those in bladder afferent neurons, and *3*) the Kv4 channel antagonist PaTx2 suppresses K_A currents in IB4 intensely stained DRG neurons but has no effect on the current in bladder afferent neurons. Thus bladder afferent and IB4-positive neurons contain the K_A current with distinct kinetic and pharmacological properties. Bladder afferent neurons appear to contain an almost exclusive, s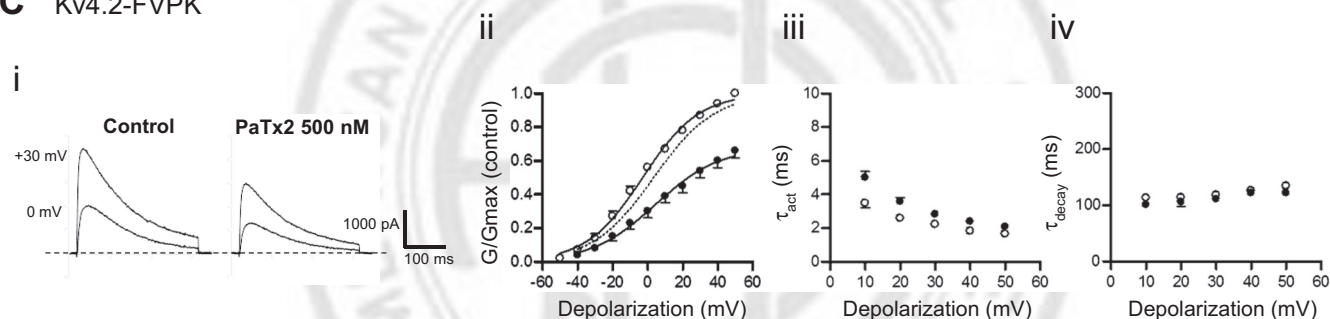low-inactivating current, whereas IB4-positive neurons contain fast-inactivating currents. The results with PaTx2 further suggest that Kv4 channels are responsible for the fast-inactivating current in IB4-positive afferents that corresponds to a subpopulation of somatic afferents.

Previous studies have shown that K_A currents in sensory neurons, including DRG cells, consist of slow- and fast-inactivating components (Akins and McCleskey 1993; Everill and Kocsis 1999; Everill et al. 1998; Gold et al. 1996; McFarlane and Cooper 1991; Yoshimura et al. 1996). Slow-inactivating K_A currents are seen in capsaicin-sensitive C-fiber afferent neurons and are sensitive to dendrotoxin, a blocker of Kv1.1- and Kv1.2-containing channels. Furthermore, Kv1.4 subunits are significant in small-sized DRG neurons, including bladder afferent neurons (Hayashi et al. 2009; Rasband et al. 2001; Takahashi et al. 2013; Yang et al. 2004). Therefore, Kv1 family channels, including the inactivating, ball-containing Kv1.4 subunit, are thought to carry the slow-inactivating K_A current in C-fiber DRG neurons. The slow kinetics and insensitivity to PaTx2 of the K_A current in bladder afferents are consistent with this idea. In contrast, the fast-inactivating K_A current is generally seen in capsaicin-insensitive, myelinated A-fiber bladder afferent neurons (Yoshimura et al. 1996). We

Fig. 6. Effects of PaTx2 on Kv4 chimeric channels. *A*: amino acid sequences of the S3 region of rat Kv4.x pore-forming subunits are aligned. A box represents the 4-amino acid portion that is diverged among the Kv4 family channels. Bold letters indicate the amino acids that were swapped between Kv4.1 and Kv4.2. *B* and *C*: data were obtained using 500 nM PaTx2, except for *B*, *ii–iv*, in which 200 nM PaTx2 was used. Open and closed circles represent before and after application of PaTx2 (*n* = 7–11). *i*: representative current traces before and after application of 500 nM PaTx2; *ii*: steady-state activation curves with broken lines generated with normalization using PaTx2 data; *iii*: τ act; and *iv*: τ decay. *D*: PaTx2 dose-dependent inhibition of Kv4 currents at 0 mV and 30 mV. *E*: steady-state inactivation.
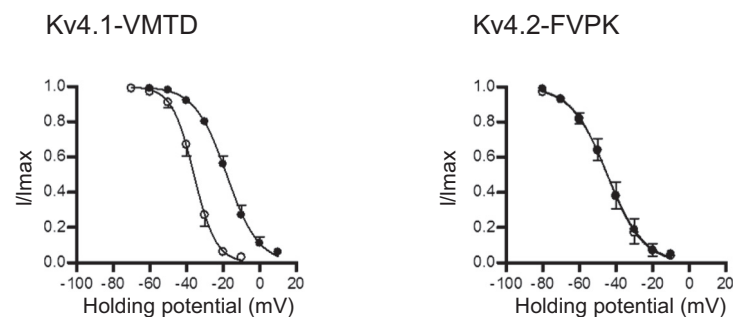
show here that IB4 intensely stained cells that respond to capsaicin exhibit prominent K_A currents with faster kinetics and PaTx2 sensitivity. Thus an IB4 intensely positive subpopulation of somatic C-fiber DRG neurons appears to possess fast-inactivating K_A currents carried by Kv4 family channels.

Voltage-Dependent and -Independent Inhibition of Kv4 Channels by PaTx2

PaTx2 is a member of gating-modifying toxins that include hanatoxins and heteropoda toxins (Diochot et al. 1999; Escoubas et al. 2002; Sanguinetti et al. 1997; Swartz and MacKinnon 1995). These toxins act on a nonpore region to exhibit voltage-dependent inhibition of target channel currents (Corzo and Escoubas 2003; Norton and Pallaghy 1998; Swartz 2007; Zarayskiy et al. 2005). With the use of heterologously expressed Kv4.x currents, we found that PaTx2 shows distinct voltage dependencies for their inhibition. The toxin altered the steady-state activation and inactivation curves of the Kv4.2 current to a positive potential. The toxin also increased time-constant values in the raising and decaying phases of Kv4.2 current. These results indicate that the inhibition of Kv4.2 current by the toxin is mainly voltage dependent. On the other hand, the toxin caused little shift in the normalized, steady-state activation and inactivation curves of the Kv4.1 current without apparent changes in the time-constant values. Therefore, the toxin inhibits the Kv4.1 current in a voltage-independent fashion. The inhibition of the Kv4.3 current by the toxin seemed to include both inhibitory mechanisms. A similar voltage-dependent and -independent blockade of Kv4.x currents has been reported recently with heteropoda toxin 2 (Desimone et al. 2011). Similar to this report, we found that four amino acids at the end of S3 primarily determine voltage dependence of the toxin action. Thus gating-modifying toxins, including PaTx2, detect subtle differences in pore-forming subunit sequences at the specific region to produce distinct inhibitory mechanisms. Hence, these toxins appear to be useful tools to identify molecular correlates of the native currents.

We took advantage of the two inhibitory fashions by PaTx2 to determine molecular correlates of the K_A current in IB4 intensely positive afferent DRG neurons that correspond to a subpopulation of somatic afferent cells. PaTx2 did not shift the normalized steady-state activation curve with minor changes in the time constants. Thus the toxin inhibition of the native K_A current is mostly voltage independent. These findings support the idea that the K_A channel in IB4 intensely stained DRG cells contains Kv4.1 and possibly Kv4.3 subunits. Our RT-PCR data are consistent with a less-obvious contribution of Kv4.2 in DRG neurons and revealed higher Kv4.1 and Kv4.3 mRNA levels in laser-captured, IB4-positive neurons than in bladder afferent neurons. There are certainly limitations in this interpretation. For instance, different Kv4.x subunits can form heteromeric channel complexes. The inhibitory fashion of heteromeric Kv4.x channel currents by the toxin might not linearly reflect the subunit composition. However, PaTx2 is considered to bind to each subunit in the tetrameric channel. Therefore, the obtained voltage-independent inhibition of the K_A current likely represents the overall subunit ratio in the native, IB4 intensely positive afferent cells.

Previous immunochemical studies indicate the presence of Kv4.3 subunits in DRG cells. For example, Kv4.3-immunore-

active proteins were found selectively in the somata of a subset of nonpeptidergic (i.e., CGRP-negative), nociceptive DRG neurons (Chien et al. 2007). Although less is known about the protein expression of the Kv4.1 subunit, due to the lack of a high-quality commercial antibody against this protein, the expression of Kv4.1 mRNA has been reported in small-sized DRG neurons using a single-cell RT-PCR method (Phuket and Covarrubias 2009). We also recently reported that Kv4.1 mRNA is expressed, not only in small-sized cells but also in all sizes of rat DRG neurons using in situ hybridization techniques (Matsuyoshi et al. 2012). Our LCM study demonstrated further that Kv4.1 and Kv4.3 mRNA expression is higher in IB4-positive afferent neurons than that in bladder afferent neurons. However, the obtained Kv4 subunit mRNA levels do not necessarily indicate the functional contribution of Kv4.1 or Kv4.3 subunits to the K_A current. In this regard, our electrophysiological data demonstrate the presence of the PaTx2-sensitive K_A current in IB4 intensely stained neurons constituting a subpopulation of somatic afferent cells. The lack of voltage-dependent inhibition of this K_A current supports a large contribution of Kv4.1 subunits to this current. It is important to note that IB4 intensely positive cells constitute a smaller fraction of somatic afferent neurons than those moderately stained. Thus further studies with IB4-negative and -moderately stained cells are needed to obtain a more complete view on molecular correlates of K_A currents between somatic and visceral afferent neurons.

In conclusion, the reduction of K^+ channel activity, including slow- and fast-inactivating K_A currents, is one of the important mechanisms for hyperexcitability and chronic pain. The present study identified that Kv4.1 and possibly Kv4.3 subunits functionally contribute to K_A channels in IB4 intensely stained neurons that correspond to a subpopulation of somatic afferent cells. Molecular correlates of K_A channels in different afferent pathways could help to develop suitable molecular targets for the treatment of pain conditions of somatic and visceral organs.

DISCLOSURES

There is no conflict of interest and no commercial affiliation in the present study. This manuscript has not been submitted to any other journals and conforms to our publication ethics guidelines.

AUTHOR CONTRIBUTIONS

Author contributions: K.T., H.M., S.N., and N.Y. conception and design of research; T.Y., K.K., Y.F., and R.T. performed experiments; T.Y. and Y.F. analyzed data; T.Y., K.T., and N.Y. interpreted results of experiments; T.Y. prepared figures; T.Y. and N.Y. drafted manuscript; K.T. and N.Y. edited and revised manuscript; K.T. and N.Y. approved final version of manuscript.

REFERENCES

Akins PT, McCleskey EW. Characterization of potassium currents in adult rat sensory neurons and modulation by opioids and cyclic AMP. *Neuroscience* 56: 759–769, 1993.

Ambalavanar R, Moritani M, Haines A, Hilton T, Dessem D. Chemical phenotypes of muscle and cutaneous afferent neurons in the rat trigeminal ganglion. *J Comp Neurol* 460: 167–179, 2003.

Bennett DL, Dmietrieva N, Priestley JV, Clary D, McMahon SB. trkA, CGRP and IB4 expression in retrogradely labelled cutaneous and visceral primary sensory neurones in the rat. *Neurosci Lett* 206: 33–36, 1996.

Birnbaum SG, Varga AW, Yuan LL, Anderson AE, Sweatt JD, Schrader LA. Structure and function of Kv4-family transient potassium channels. *Physiol Rev* 84: 803–833, 2004.

Chien LY, Cheng JK, Chu D, Cheng CF, Tsaur ML. Reduced expression of A-type potassium channels in primary sensory neurons induces mechanical hypersensitivity. *J Neurosci* 27: 9855–9865, 2007.

Corzo G, Escoubas P. Pharmacologically active spider peptide toxins. *Cell Mol Life Sci* 60: 2409–2426, 2003.

Dang K, Bielefeldt K, Gebhart GF. Differential responses of bladder lumbosacral and thoracolumbar dorsal root ganglion neurons to purinergic agonists, protons, and capsaicin. *J Neurosci* 25: 3973–3984, 2005.

Desimone CV, Zarayskiy VV, Bondarenko VE, Morales MJ. Heteropoda toxin 2 interaction with Kv4.3 and Kv4.1 reveals differences in gating modification. *Mol Pharmacol* 80: 345–355, 2011.

Diochot S, Drici MD, Moinier D, Fink M, Lazdunski M. Effects of phrixotoxins on the Kv4 family of potassium channels and implications for the role of Ito1 in cardiac electrogenesis. *Br J Pharmacol* 126: 251–263, 1999.

Escoubas P, Diochot S, Célérier ML, Nakajima T, Lazdunski M. Novel tarantula toxins for subtypes of voltage-dependent potassium channels in the Kv2 and Kv4 subfamilies. *Mol Pharmacol* 62: 48–57, 2002.

Everill B, Kocsis JD. Reduction in potassium currents in identified cutaneous afferent dorsal root ganglion neurons after axotomy. *J Neurophysiol* 82: 700–708, 1999.

Everill B, Rizzo MA, Kocsis JD. Morphologically identified cutaneous afferent DRG neurons express three different potassium currents in varying proportions. *J Neurophysiol* 79: 1814–1824, 1998.

Gold MS, Shuster MJ, Levine JD. Characterization of six voltage-gated K$^+$ currents in adult rat sensory neurons. *J Neurophysiol* 75: 2629–2646, 1996.

Hall A, Stow J, Sorensen R, Dolly JO, Owen D. Blockade by dendrotoxin homologues of voltage-dependent K$^+$ currents in cultured sensory neurones from neonatal rats. *Br J Pharmacol* 113: 959–967, 1994.

Hayashi Y, Takimoto K, Chancellor MB, Erickson KA, Erickson VL, Kirimoto T, Nakano K, de Groat WC, Yoshimura N. Bladder hyperactivity and increased excitability of bladder afferent neurons associated with reduced expression of Kv1.4 alpha-subunit in rats with cystitis. *Am J Physiol Regul Integr Comp Physiol* 296: R1661–R1670, 2009.

Hwang SJ, Oh JM, Valtschanoff JG. Expression of the vanilloid receptor TRPV1 in rat dorsal root ganglion neurons supports different roles of the receptor in visceral and cutaneous afferents. *Brain Res* 1047: 261–266, 2005.

Ivanavicius SP, Blake DR, Chessell IP, Mapp PI. Isolectin B4 binding neurons are not present in the rat knee joint. *Neuroscience* 128: 555–560, 2004.

Kostyuk PG, Veselovsky NS, Fedulova SA, Tsyndrenko AY. Ionic currents in the somatic membrane of rat dorsal root ganglion neurons-III. Potassium currents. *Neuroscience* 6: 2439–2444, 1981.

Lu J, Zhou XF, Rush RA. Small primary sensory neurons innervating epidermis and viscera display differential phenotype in the adult rat. *Neurosci Res* 41: 355–363, 2001.

Matsuyoshi H, Takimoto K, Yunoki T, Erickson VL, Tyagi P, Hirao Y, Wanaka A, Yoshimura N. Distinct cellular distributions of Kv4 poreforming and auxiliary subunits in rat dorsal root ganglion neurons. *Life Sci* 91: 258–263, 2012.

McFarlane S, Cooper E. Kinetics and voltage dependence of A-type currents on neonatal rat sensory neurons. *J Neurophysiol* 66: 1380–1391, 1991.

Nagy JI, Hunt SP. Fluoride-resistant acid phosphatase-containing neurones in dorsal root ganglia are separate from those containing substance P or somatostatin. *Neuroscience* 7: 89–97, 1982.

Norton RS, Pallaghy PK. The cystine knot structure of ion channel toxins and related polypeptides. *Toxicon* 36: 1573–1583, 1998.

Phuket TR, Covarrubias M. Kv4 channels underlie the subthreshold-operating A-type K-current in nociceptive dorsal root ganglion neurons. *Front Mol Neurosci* 2: 3, 2009.

Rasband MN, Park EW, Vanderah TW, Lai J, Porreca F, Trimmer JS. Distinct potassium channels on pain-sensing neurons. *Proc Natl Acad Sci USA* 98: 13373–13378, 2001.

Ren X, Shand SH, Takimoto K. Effective association of Kv channel-interacting proteins with Kv4 channel is mediated with their unique core peptide. *J Biol Chem* 278: 43564–43570, 2003.

Sanguinetti MC, Johnson JH, Hammerland LG, Kelbaugh PR, Volkmann RA, Saccomano NA, Mueller AL. Heteropodatoxins: peptides isolated from spider venom that block Kv4.2 potassium channels. *Mol Pharmacol* 51: 491–498, 1997.

Silverman JD, Kruger L. Selective neuronal glycoconjugate expression in sensory and autonomic ganglia: relation to lectin reactivity to peptide and enzyme markers. *J Neurocytol* 19: 789–801, 1990.

Swartz KJ. Tarantula toxins interacting with voltage sensors in potassium channels. *Toxicon* 49: 213–230, 2007.

Swartz KJ, MacKinnon R. An inhibitor of the Kv2.1 potassium channel isolated from the venom of a Chilean tarantula. *Neuron* 15: 941–949, 1995.

Takahashi R, Yoshizawa T, Yunoki T, Tyagi P, Naito S, de Groat WC, Yoshimura N. Hyperexcitability of bladder afferent neurons associated with reduction of Kv1.4 α-subunit in rats with spinal cord injury. *J Urol* 190: 2296–2304, 2013.

Takimoto K, Hayashi Y, Ren X, Yoshimura N. Species and tissue differences in the expression of DPPY splicing variants. *Biochem Biophys Res Commun* 348: 1094–1100, 2006.

Takimoto K, Li D, Hershman KM, Li P, Jackson EK, Levitan ES. Decreased expression of Kv4.2 and novel Kv43 K$^+$ channel subunit mRNAs in ventricles of renovascular hypertensive rats. *Circ Res* 81: 533–539, 1997.

Takimoto K, Yang EK, Conforti L. Palmitoylation of KChIP splicing variants is required for efficient cell surface expression of Kv4.3 channels. *J Biol Chem* 277: 26904–26911, 2002.

Vydyanathan A, Wu ZZ, Chen SR, Pan HL. A-type voltage-gated K$^+$ currents influence firing properties of isolectin B4-positive but not isolectin B4-negative primary sensory neurons. *J Neurophysiol* 93: 3401–3409, 2005.

Yang EK, Takimoto K, Hayashi Y, de Groat WC, Yoshimura N. Altered expression of potassium channel subunit mRNA and α-dendrotoxin sensitivity of potassium currents in rat dorsal root ganglion neurons after axotomy. *Neuroscience* 123: 867–874, 2004.

Yoshimura N, de Groat WC. Increased excitability of afferent neurons innervating rat urinary bladder after chronic bladder inflammation. *J Neurosci* 19: 4644–4653, 1999.

Yoshimura N, Seki S, Erickson KA, Erickson VL, Chancellor MB, de Groat WC. Histological and electrical properties of rat dorsal root ganglion neurons innervating the lower urinary tract. *J Neurosci* 23: 4355–4361, 2003.

Yoshimura N, White G, Weight FF, de Groat WC. Different types of Na$^+$ and A-type K$^+$ currents in dorsal root ganglion neurones innervating the rat urinary bladder. *J Physiol* 494: 1–16, 1996.

Zarayskiy VV, Balasubramanian G, Bondarenko VE, Morales MJ. Heteropoda toxin 2 is a gating modifier toxin specific for voltage-gated K$^+$ channels of the Kv4 family. *Toxicon* 45: 431–442, 2005.

MP76-01

Investigation into Constitutive Expression of Nerve Growth Factor in Bladder By Bladder wall Injection of NGF Antisense

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INTRODUCTION AND OBJECTIVES: Overexpression of nerve growth factor NGF in bladder has been established as a key molecular mediator in the lower urinary tract symptoms. Overactive bladder patients responding to intradetrusor injection of botulinum toxin showed a reduced NGF expression in histological specimens. In our previous study, bladder instillation of NGF antisense was able to directly suppress the inducible NGF expression in urothelium following acetic acid insult, but the effect of intravesical treatment on the detrusor expression of NGF was inconclusive. Therefore in the present study, we explored the direct effect of bladder wall injection of NGF antisense on the NGF expression in different regions of the bladder.

METHODS: Adult female Sprague-Dawley rats were given bladder wall injection (10-20µL) of either saline (sham) (n=3) or NGF antisense (0.1-10µM) complexed with liposomes under isoflurane anesthesia(n=6). Total volume of injection was split into four sites around the bladder. Abdomen was sutured back after surgery and animal returned to cages. 48h after injection, open transurethral cystometry under urethane anaesthesia (dose 1g/kg, subcutaneously) was performed with saline infused at 0.04ml/min. Bladder was harvested following sacrifice for measuring NGF protein levels by ELISA.

RESULTS: Cystometric parameters were indistinct between sham and the treated groups. The measured tissue specific NGF levels were normalized to the respective protein concentration and expressed as pg/mg of protein. There was insignificant difference in the NGF levels of two tissue regions from sham group with 295.8± 10.26pg/mg in urothelium and 273.9± 46.28pg/mg in detrusor(n=3). In contrast, bladder wall injection of NGF antisense reduced the constitutive production of NGF in both urothelium and detrusor regions. Paired analysis of NGF levels in urothelium and detrusor for the antisense treated group found significant reduction in detrusor relative to the higher levels in urothelium 262.0± 15.76pg/mg vs 286.8± 23.28pg/mg, respectively ($p < 0.05$).

CONCLUSIONS: Gene silencing of NGF gene in detrusor following bladder wall injection supports the intradetrusor injection of NGF antisense complexed with liposome as a potential treatment for refractory overactive bladder associated with NGF overexpression in bladder smooth muscle. NGF antisense is able to suppress both constitutive and inducible expression of NGF in bladder, and inducible NGF expression has implications in the OAB pathophysiology.

PD9-01
THERAPEUTIC EFFECT OF ANTISENSE OLOGONUCLEOTIDE (OND) TARGETING NERVE GROWTH FACTOR (NGF) ON BLADDER HYPESENSITIVITY IN RAT WITH EXPERIMENTAL COLITIS

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INTRODUCTION AND OBJECTIVES: Pelvic organ "cross sensitization" seems to contribute to overlapping symptoms in patients with chronic pelvic pain syndrome (CPPS) including bladder pain syndrome/interstitial cystitis (BPS/IC). However, it is not known whether NGF overexpression in the bladder is involved in this cross sensitization mechanism. Therefore, we investigated whether local instillation of liposome-OND conjugates (L-OND) targeting NGF overexpression in the bladder can suppress bladder hypersensitivity in a rat model of experimental colitis.

METHODS: Five groups of female SD rats were used; (a) control (no treatment), (b) colitis-OND (intracolonic 2,4,6,trinitrobenzen sulfonic acid [TNBS] enema & intravesical L-OND) , (c) colitis-saline (TNBS & intravesical saline), (d) sham-OND (L-OND was given without colitis) and (e) sham-saline (saline was given without colitis). A day before evoking TNBS colitis, either L-OND or saline was instilled to the bladder. (1) Behavior testing: Resiniferatoxin (0.3μM, 0.3 ml) was administered into the bladder for 1 min to evaluate nociceptive behaviors such as licking (lower abdominal licking) and freezing (motionless head-turning) for 15 min. (2) Cystometry: Saline followed by 0.1% acetic acid (AA) were continuously infused to evaluate changes in intracontraction intervals (ICIs) in conscious rats. (3) Molecular analysis: Harvested bladders were divided to mucosal and detrusor layers to measure the mRNA and protein expression of NGF by qPCR and ELISA, respectively.

RESULTS: (1) In the colitis-saline group, the score of freezing behavior, which represents bladder pain sensation, was significantly higher than that of other groups including the colitis-OND group. (2) The reduction rate of ICI after AA instillation in the colitis-saline group was approximately 40% in contrast to 10% in the colitis-OND group. (3) The mRNA and protein expression of NGF in the mucosa was significantly higher in the colitis-saline group compared to the colitis-OND group.

CONCLUSIONS: These results indicate that intravesical treatment of NGF antisense with liposome, which suppressed mucosal NGF overexpression, reduced colitis-induced bladder hypersensitivity evidenced by increased bladder pain behavior (freezing) and AA-induced bladder overactivity. Thus, the intravesical NGF antisense treatment could be effective for hypersensitive bladder symptoms in CPPS including BPS/IC, in which the cross-sensitization mechanism contributes to overlapping symptoms from different pelvic organs.

MP1-17

Therapeutic effects of intravesical application of liposome conjugated with antisense oligonucleotide targeting nerve growth factor on neurogenic detrusor overactivity in rats with spinal cord injury

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INTRODUCTION AND OBJECTIVES: We recently reported that instillation of liposome conjugated with antisense oligonucleotide (OND) targeting nerve growth factor (NGF) into the bladder suppressed bladder overactivity in a rat model of acute cystitis (Kashyap et al., J Urol, 2013). Therefore, this study investigated whether instillation of liposome with NGF-targeting antisense OND into the bladder can suppress neurogenic detrusor overactivity shown by non-voiding contractions (NVCs) during the storage phase in rats with spinal cord injury (SCI).

METHODS: Adult female Sprague-Dawley rats were used. SCI was produced by complete transection of the Th8-9 spinal cord. After 3 weeks, SCI rats were divided into three groups; (a) Control group (no treatment), (b) Liposome without antisense OND group, (c) Liposome with NGF-targeting antisense OND group, in which. NGF antisense-liposome solution (1 ml) containing $6\mu l$ of anti-NGF oligonucleotide (2mM) in $994\mu l$ of liposome (7mM) was injected into the bladder and retained for 30 min. At 4 weeks after SCI, awake cystometry was performed during continuous infusion of saline (0.08 ml/min) into the bladder. Cystometric parameters evaluated included the amplitude (cmH_2O) and frequency (number/min) of NVCs, maximal voiding pressure (MVP) and residual urine volume (RV).

RESULTS: There was no any significance in the amplitude and frequency of NVCs between control (no treatment) and liposome without antisense OND groups of SCI rats. However, in the liposome with NGF-targeting antisense OND group, the amplitude and frequency of NVCs were significantly reduced compared to control (no treatment) (38 and 55% reductions, respectively) and liposome without antisense OND groups (35 and 58% reductions, respectively) of SCI rats. MVP and RV were also significantly reduced in the NGF-targeting antisense OND group compared to other groups.

CONCLUSIONS: These results indicate that intravesical treatment with liposome conjugated with antisense OND targeting NGF effectively reduces neurogenic detrusor overactivity as evidenced by the reduction of NVCs in SCI rats. Because NGF upregulation in the bladder is shown to be one of the key mechanisms inducing bladder overactivity, the intravesical liposome-NGF antisense delivery could be a novel option for the treatment of neurogenic detrusor overactivity in SCI.

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LIPOSOME-BASED INTRAVESICAL THERAPY TARGETING NERVE GROWTH FACTOR (NGF) AMELIORATES BLADDER HYPERSENSITIVITY IN RATS WITH EXPERIMENTAL COLITIS

Hypothesis / aims of study
The complex pathophysiology of chronic pelvic pain syndrome (CPPS) and bladder pain syndrome/interstitial cystitis (BPS/IC) could be interrelated. It has recently been proposed that pelvic organ "cross sensitization" contributes to the clinically overlapping symptoms in CPPS such as irritable bowel syndrome (IBS) and BPS/IC. Previous animal studies also demonstrated that experimental colitis evokes bladder overactivity evidenced by frequent urination in association with hyperexcitability of afferent neurons innervating the bladder [1] although it has not been investigated whether this colitis model exhibits an increase in bladder pain sensation. Meanwhile, overexpression of nerve growth factor (NGF) in the bladder is thought to be one of the key factors in the symptom development in BPS/IC patients.   We recently reported that instillation of liposome conjugated with antisense oligonucleotide (OND) targeting NGF into the bladder suppressed bladder overactivity in a rat model of acute cystitis [2]. Therefore, this study was planned to explore whether bladder hypersensitivity induced by experimental colitis and NGF overexpression in the bladder are induced after colitis and whether intravesical liposomal-OND treatment can suppress bladder hypersensitivity and NGF expression in a rat model with experimental colitis.

Study design, materials and methods
- Animal groups: Adult female Sprague-Dawley rats were used; (a) control group (no treatment), (b) colitis-OND group (intracolonic 2,4,6,trinitrobenzen sulfonic acid [TNBS] enema and intravesical liposomal OND were given) , (c) colitis-saline group (intracolonic TNBS and intravesical saline were given), (d) sham-OND group (intravesical liposomal OND was given without colitis) and (e) sham-saline group (intravesical saline was given without colitis).
- Intravesical administration of NGF antisense-liposome solution (liposomal-OND): Under isoflurane anesthesia, 0.2ml of either liposomal-OND or saline was instilled to the bladder through an inserted urethral catheter.
- Experimental colitis model: Twenty-four hours after instillation of liposomal-OND or saline and fasting, colitis was induced by the enema of 30mg TNBS dissolved in 50% ethanol through a polyethylene catheter inserted 8 cm proximal to the anus in a head-down position. Ten days after liposomal-OND or saline injection, animals were subjected to either in vivo studies or bladder tissue removal.

(1) Nociceptive behaviour testing: Licking (lower abdominal licking) and freezing behaviours (motionless head-turning towards lower abdomen) in response to 1-min intravesical administration of resiniferatoxin (RTX), a TRPV1 receptor agonist, were examined. After 2 hours acclimation in a metabolic cage, RTX (0.3µM, 0.3ml) was instilled through an inserted urethral catheter for 1 min, and the catheter was then removed. Thereafter, both licking and freezing behaviours were scored during 5-s intervals for 15 minutes in the cage (n=4-6).
(2) Awake cyctometry: Intravesical catheters were implanted under urethane anaesthesia 3 days before cystometry. PE-50 catheter with the end flared by heat was inserted into the bladder dome, ligated and placed subcutaneously. Saline followed by 0.1% acetic acid (AA) were continuously infused to evaluate changes in intracontraction intervals (ICIs) in conscious rats.
(3) Immunohistochemistry: The frozen section of the bladder was stained with NGF antibody (1:250 dilution). The positive staining was visualized with a DAB kit.
(4) Molecular analysis of NGF: The harvested bladder was micro-dissected to divide into mucosal and detrusor layers. Quantitative polymerase chain reaction (qPCR) and Enzyme-Linked ImmunoSorbent Assay (ELISA) were used to measure the mRNA and protein expression of NGF, respectively (n=3-5).

Results
(1) In the colitis-saline group, the score of freezing behaviour was significantly higher than that of all other groups including the colitis-OND group (Figure 1). The score of licking behaviour in the colitis-saline group was significantly higher than in the control group and tended to higher compared to other 3 groups without significant differences. (2) ICIs before intravesical AA stimulation were not different among groups; however, the ICI reduction rate after AA instillation into the bladder was significantly higher in the colitis-saline group than that in the colitis-OND group (Figure 2). (3) There was increased immunoreactivity of NGF in the bladder mucosa in the colitis-saline group, whereas there was only faint staining in the control and colitis-OND groups. (4) The mRNA expression of NGF in the colitis-saline group was significantly increased in the mucosa compared to control and colitis-OND groups (Figure 3). In addition the protein level of NGF in the mucosa was also higher in the colitis-saline group compared to other groups.
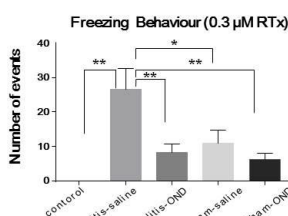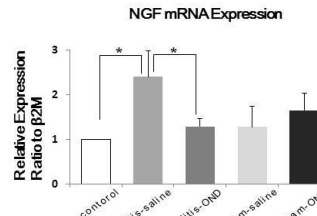

(Figure 1) Freezing behaviour was significantly increased in the colitis-saline group and decreased in the colitis-OND group($*p$<0.05, $**p$< 0.01).
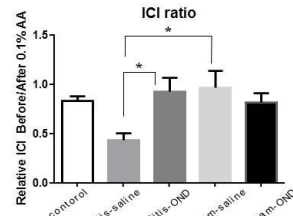
(Figure 2) The ICI reduction rate after intravesical infusion of acetic acid (AA) was significantly higher in the colitis-saline group than that in the colitis-OND group ($*p$<0.05).

(Figure 3) Expression of NGF mRNA was significantly increased in the colitis-saline group compared to control and colitis-OND groups ($*p$<0.05).

Interpretation of results
These results indicate that: (1) colitis evoked by TNBS induced bladder hypersensitivity shown by increased freezing behaviour, which represents bladder pain sensation, and enhanced bladder overactivity in response to nociceptive bladder stimuli such as RTX or AA and (2) colitis-induced bladder hypersensitivity is associated with an increase of NGF expression at both mRNA and protein levels in the bladder mucosa. Furthermore, the intravesical liposome-based treatment with NGF antisense, which reduced the mucosal expression of NGF, had a therapeutic effect on colitis-induced bladder hypersensitivity as evidenced by reductions in RTX-induced freezing behaviour and AA-induced bladder overactivity. Taken together, it is likely that NGF overexpression in the bladder mucosa plays an important role in the colon-to-bladder cross-sensitization to induce bladder hypersensitivity after colitis and that intravesical application of liposomal OND targeting NGF is effective to reduce bladder pain and overactivity induced by colitis.

Concluding message
This study shows that the rat model of experimental colitis is useful to study the mechanism inducing bladder hypersensitivity such as pain behaviour in addition to changes in bladder activity. The liposome-based antisense treatment targeting NGF in the bladder could be a new, effective modality for the treatment of bladder pain and overactivity in CPPS patients including those with BPS/IC and IBS, in whom the pelvic organ "cross sensitization" mechanism is involved in overlapping symptoms from different pelvic organs.

References
1. Michael A.Pezzone, et al. Gastroenterology. 2005 Jun;128(7):1953-64
2. M.Kashyap, et al. J of Urol. 2013 Aug;192(2):157-64